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Polyhydroxyalkanoates production by photosynthetic mixed cultures

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Para o meu Pai

e para a minha Mãe

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Abstract

Polyhydroxyalkanoates (PHAs) are natural biologically synthesized polymers that have been the subject of much interest in the last decades due to their biodegradability. Thus far, its microbial production is associated with high operational costs, which increases PHA prices and limits its marketability. To address this situation, this thesis' work proposes the utilization of photosynthetic mixed cultures (PMC) as a new PHA production system that may lead to a reduction in operational costs. In fact, the operational strategies developed in this work led to the selection of PHA accumulating PMCs that, unlike the traditional mixed microbial cultures, do not require aeration, thus permitting savings in this significant operational cost. In particular, the first PHA accumulating PMC tested in this work was selected under non-aerated illuminated conditions in a feast and famine regime, being obtained a consortium of bacteria and algae, where photosynthetic bacteria accumulated PHA during the feast phase and consumed it for growth during the famine phase, using the oxygen produced by algae. In this symbiotic system, a maximum PHA content of 20% cell dry weight (cdw) was reached, proving for the first time, the capacity of a PMC to accumulate PHA. During adaptation to dark/light alternating conditions, the culture decreased its algae content but maintained its viability, achieving a PHA content of 30% cdw. Also, the PMC was found to be able to utilize different volatile fatty acids for PHA production, accumulating up to 20% cdw of a PHA co-polymer composed of 3-hydroxybutyrate (3HB) and 3-hydroxyvalerate (HV) monomers. Finally, a new selective approach for the enrichment of PMCs in PHA accumulating bacteria was tested. Instead of imposing a feast and famine regime, a permanent feast regime was used, thus selecting a PMC that was capable of simultaneously growing and accumulating PHA, being attained a maximum PHA content of 60% cdw, the highest value reported for a PMC thus far. The results presented in this thesis prospect the utilization of cheap, VFA-rich fermented wastes as substrates for PHA production, which combined with this new photosynthetic technology opens up the possibility for direct sunlight illumination, leading to a more cost-effective and environmentally sustainable PHA production process.

Keywords: Polyhydroxyalkanoates (PHA); photosynthetic mixed cultures (PMC); feast and famine regime; dark/light cycles; volatile fatty acids (VFA); permanent feast regime.

Resumo

Polihidroxialcanoatos (PHAs) são polímeros naturais sintetizados biologicamente que nas últimas décadas têm sido alvo de grande interesse devido à sua biodegradabilidade. Actualmente os PHAs são produzidos microbiologicamente com custos operacionais elevados que inflaccionam o seu preço e limitam a sua comercialização. Em resposta a esta situação, o trabalho desenvolvido nesta tese propõe a utilização de culturas mistas fotossintéticas (CMF) como um novo sistema de produção de PHAs que poderá levar a uma redução dos custos operacionais. De facto, as estratégias operacionais desenvolvidas neste trabalho levaram à selecção de CMFs acumuladoras de PHA que, ao contrário das culturas mistas tradicionais, não necessitam de arejamento, permitindo uma redução de custos em relação a este aspecto. Em concreto, a primeira CMF acumuladora de PHAs que foi testada neste trabalho foi seleccionada através de um regime de fome e fartura na presença de luz e sem arejamento, tendo-se obtido um consórcio de bactérias e algas em que as bactérias fotossintéticas acumulavam PHAs durante a fase de fartura e consumiam-no para crescimento durante a fase de fome, usando, para tal, o oxigénio produzido pelas algas. Neste sistema simbiótico foi possível alcançar um conteúdo máximo em PHA de 20% em peso seco celular (psc), demonstrando, pela primeira vez, a capacidade de acumulação de PHA de uma CMF. Durante a adaptação para condições alternadas luz/escuro, ocorreu uma diminuição na quantidade de algas da cultura, que manteve no entanto a sua viabilidade, atingindo um conteúdo em PHA de 30% psc. Verificou-se também que a CMF poderia utilizar diferentes ácidos gordos voláteis (AGVs) para produção de PHA, conseguindo acumular até 20% psc de um co-polímero de PHA composto por monómeros de 3-hidroxibutiraro (3HB) e 3-hidroxivalerato (HV). Por último, testou-se um novo método de selecção para enriquecimento de CMFs em bactérias acumuladoras de PHA. Em vez de se impor um regime de fome e fartura, utilizou-se um regime de fartura permanente, selecionando-se deste modo uma CMF que era capaz de crescer e acumular PHA simultaneamente, tendo-se alcançado um conteúdo máximo em PHA de 60% psc, o maior valor descrito até ao momento para CMFs. Os resultados apresentados neste trabalho prenunciam a utilização de resíduos baratos fermentados, ricos em AGVs, como substratos para produção de PHAs, o que combinado com a nova tecnologia fotossintética proposta neste trabalho, abrem a possibilidade de uma utilização directa de iluminação solar, levando à criação de um processo de produção de PHAs menos oneroso e mais ambientalmente sustentável.

Termos-chave: Polihidroxialcanoatos (PHA); culturas mistas fotossintéticas (CMF); regime de fome e fartura; ciclos de luz/escuro; ácidos gordos voláteis (AGV); regime fartura permanente.

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3H2MB	3-hydroxy-2-methylbutyrate
3H2MV	3-hydroxy-2-valerate
3HB	3-hydroxybutyrate
3HHx	3-hydroxyhexanoate
3HV	3-hydroxyvalerate
4HB	4-hydroxybutyryl
ADF	Aerobic Dynamic Feeding
ATP	Adenosine Triphosphate
Cdw	Cell dry weight
CoA	Coenzime A
D	Dextrorotatory
EBPR	Enhanced Biological Phosphorus Removal
F/F	Feast to Famine ratio
FF	Feast and Famine
FISH	Fluorescence in situ Hybridisation
GAO	Glycogen Accumulating Organisms
Gly	Glycogen
HPLC	High-Performance Liquid Chromatography
HRT	Hydraulic Retention Time
k_p	Kinetic coefficient of PHB consumption
L	Levorotary

MCL	Medium-Chain-Length
MMC	Mixed Microbial Cultures
NADH	Nicotinamide Adenine Dinucleotide
NADPH	Nicotinamide Adenine Dinucleotide Phosphate
OLR	Organic Loading Rate
P(3HB-3HV)	Poly(3- hydroxybutyrate-co-3-hydroxyvalerate)
P3HB	Poly(3-hydroxybutyrate)
P3HV	Poly(3-hydroxyvalerate)
PAO	Polyphosphate Accumulating Organisms
PCL	Poly(ε-caprolactone)
PHA	Polyhydroxyalkanoate
PLA	Polylactic acid
PMC	Photosynthetic Mixed Culture
PNS	Purple Non-Sulfur
PVA	Polyvinyl alcohol
q_{Carbs}	Maximum specific carbohydrate utilization rate
q_P	Maximum specific PHA production rate
$-q_{PO4}$	Maximum specific phosphate consumption rate
$-q_S$	Maximum specific substrate uptake rate
SBR	Sequencing Batch Reactor
SCL	Short-Chain- Length
SRT	Sludge Retention Time
TCA	Tricarboxylic Acid Cycle
VFA	Volatile Fatty Acid

VSS	Volatile Suspended Solids
X	Active biomass
$Y_{\text{Carbs/PHA}}$	Yield of carbohydrates per PHA consumed
$Y_{\text{Carbs/S}}$	Yield of carbohydrates per substrate consumed
$Y_{\text{PHA/Carbs}}$	Yield of PHA per carbohydrates consumed
$Y_{\text{PHA/S}}$	Yield of PHA per substrate consumed
$Y_{X/\text{S}}$	Yield of biomass per substrate consumed
μ	Specific growth rate

"When I was a child, I used to go with my mother to the supermarket and she would always take with her, her own cloth bag to carry groceries home. Back then, plastic bags were rare and many products were conditioned in glass jars, card boxes and cans. But in few years, plastic became easily accessible and industries rapidly adopted this material. Soon, plastic bags were being given at every shop and consumers could take as many as they wanted…

Plastics are now everywhere: cars are no longer heavy and solely made of iron, but are lighter and faster… We have flat screen televisions, smartphones, tablets… We have plastic prostheses, hearing devices, new medical tools… Plastic became an essential commodity that increased societies' comfort and quality of life. However, we cannot look just to the bright side of the coin."

1

Introduction

1.1 PLASTICS

Plastics are polymeric materials with versatile qualities of strength, lightness, resistance and durability that can be molded into a variety of products (Hopewell et al., 2009). They can be used in innumerous applications, and as a result, the plastic industry has been growing almost 5% per year in the last 20 years. In 2012, the global plastic production was estimated at 290 million tons (PlasticsEurope, 2013). This is a market with sales evaluated in 300 billion Euros in Europe alone, where the plastics demand reached 46 million tons in 2012 (PlasticsEurope, 2013). Most of these were used for packaging (39%) and building & construction (20%) (Figure 1.1).

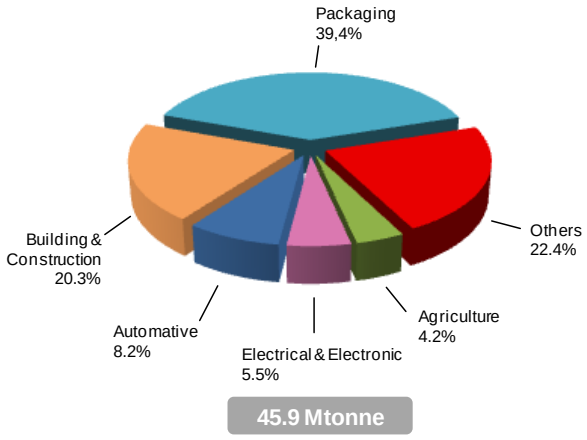

Figure 1.1 – European plastics demand by segment in 2012 (PlasticsEurope, 2013).

Though a considerable amount of the plastics was used in long-life applications, 25 million tons of plastics ended up in the waste stream. A great part of these were still recovered for recycling (26%) and energy production (36%), but more than one third (38%) was eventually disposed in landfills (PlasticsEurope, 2013). Worldwide, it is expected that a much higher percentage of plastics are discarded and directly disposed in nature. Moreover, most types of plastics are nondegradable and even the degradable ones may persist for a considerable time, accumulating in the natural environment and contaminating soils and water resources (Hopewell et al., 2009). This plastic pollution has become a worldwide problem, causing harmful effects not only in human populations but especially, in the animal kingdom (Figure 1.2).

Figure 1.2 – Plastic pollution. A – Devotees cross the polluted waters of the Ganges river, April 2013 (AFP, 2013). B – Red-eared slider turtle, found with a discarded plastic around her shell (CCM, 2010)

Furthermore, concerns with environmental sustainability are not restricted to the final end-use of plastics. They extend back to the initial production steps of the plastic. Indeed, most of the currently produced plastics are derived from petrochemicals that are produced from fossil oil and gas, which present continuously rising prices that fluctuate at each geo-political crisis. Additionally, these resources are also known for their contribution to environmental pollution. In fact, not only can petrochemicals have a devastating effect during oil spills, but they are also important contributors to global warming during combustion for energy production (Houghton, 2005). Another aspect of the petrochemical-based plastics is that the plastic industry takes up an important part of the extracted fossil resources. Around 4% of the world oil and gas production is used as feedstock for plastics and a further 3 – 4% is expended to provide energy for their manufacturing (Hopewell et al., 2009). These resources are finite and will become depleted, which added to the plastic related pollution observed in the natural habitats indicates that the current production of plastics is not environmentally sustainable.

A positive note is that society is becoming more aware of this problem and the growing public concern regarding the environment, climate change and fossil fuel resources is pressing governments, companies, and researchers to discover and promote sustainable alternatives to petrochemical-based plastics. This will necessarily imply a compromise between the utilization of new and environmentally sustainable feedstocks, and the production of biodegradable plastics.

1.2 BIODEGRADABLE PLASTICS

By definition, biodegradable plastics are polymeric materials that can be decomposed through the action of enzymes and chemicals associated with living organisms and/or through abiotic reactions, like photodegradation, oxidation and hydrolysis (Albertsson and Karlsson, 1994).

Great interest has been placed in the development of these plastics in an attempt to address societies' request for more ecological biopolymers. Consequently, several materials have surged as candidates for the production of biodegradable plastics, but thus far, they have only been used in very specific areas, like in the medical field (e.g. drug delivery applications, surgical implants), in agriculture (e.g. mulch fields) and in packaging (Amass et al., 1998).

The polymeric materials currently used in the production of biodegradable plastics can be divided into three categories: chemically synthesized polymers, starch-based biodegradable materials and polyhydroxyalkanoates (PHAs) (Mumtaz et al., 2010).

The first category includes for example polylactic acid (PLA), poly(ε-caprolactone) (PCL) and polyvinyl alcohol (PVA). All these polymers are currently used for biodegradable plastics production (Nair and Laurencin, 2007). However, they don't comprise all the properties of conventional plastics and their utilization is limited to specific applications (Khanna and Srivastava, 2005). Therefore, they are not a viable alternative for a large-scale replacement of traditional plastics.

In relation to the starch-based plastics, these materials are produced by melting starch and blending it on a molecular level with a conventional plastic. The starch fraction remains accessible for microbial degradation and the plastic becomes porous after the starch is degraded. These pores can then facilitate further plastic degradation (Chandra and Rustgi, 1998). Although this strategy can be used to reduce the polymer degradation time, the petrochemical fraction of the plastic will still require a long degradation period (Khanna and Srivastava, 2005).

In the case of PHAs, they are natural polyesters produced by many different microorganisms (Chandra and Rustgi, 1998). They can be synthesized from renewable resources and because they are completely biodegradable biopolymers, they compromise all the ecological requirements for the production of sustainable biodegradable plastics. Also, they present physico-chemical characteristics similar to conventional plastics, which makes them a viable alternative to the petrochemical-based plastics.

1.3 POLYHYDROXYALKANOATES

Polyhydroxyalkanoates (PHAs) are biopolymers synthesized by numerous microorganisms and internally accumulated as carbon and energy reserves. This accumulation occurs in the presence of excess carbon and, in most of the cases, under growth limiting conditions and leads to the formation of intracellular PHA granules. Figure 1.3 shows the general structure of PHAs.

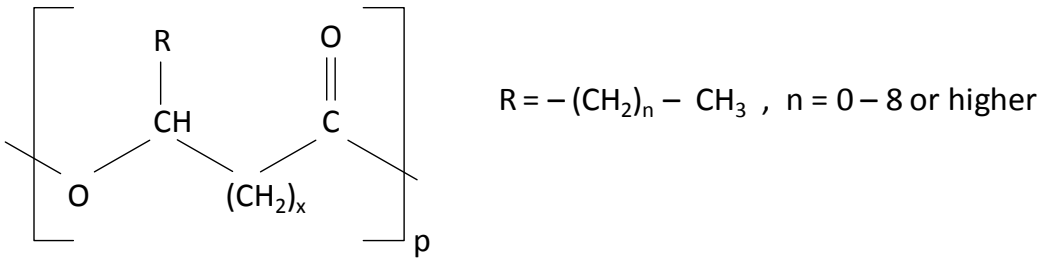

Figure 1.3 – General structure of PHAs. p – Number of monomers in the polymer; x – Variable number of carbon atoms in the linear structure; R – alkyl radical; n – Variable number of carbon atoms in the alkyl radical.

These polyesters are composed of a linear carbon branch with variable size, but that typically varies from 3 to 4 carbons (x = 1 and 2, respectively), and an alkyl radical, R, that can incorporate more than 9 carbons in the case of longer alkyl substitutes (Chandra and Rustgi, 1998). Depending on the number of constituent carbon atoms of the monomer units, PHAs can be divided in two groups. They can be short-chain- length (SCL) polymers with monomers containing 3 – 5 carbon atoms, or medium-chain-length (MCL) with monomers containing 6 – 18 carbon atoms (Philip, et al., 2007). With such a variety of sizes and possibilities, more than 150 different monomer units have already been observed in biologically formed PHA. However, the PHAs that are most commonly produced by bacteria are the homopolymer poly(3-hydroxybutyrate) (P3HB) and the co-polymer poly(3- hydroxybutyrate-co-3-hydroxyvalerate) (P(3HB-3HV)) (Laycock et al., 2013).

P3HB is a SCL polymer that presents mechanical properties comparable to polypropylene (Table 1.1), which is an important aspect since polypropylene is one of the most important plastics used worldwide and that presents an average annual production growth rate of 4.4% (Ceresana, 2012).

Table 1.1 – P3HB and polypropylene physico-chemical properties (Amass et al., 1998; Laycock et al., 2013).

Property	P3HB	Polypropylene
Molecular weight (g/mol)	5×10^5	2×10^5
Crystallinity (%)	80	70
Melting temperature (°C)	175	176
Glass transition temperature (°C)	5	- 10
Tensile strength (MPa)	37	38
Extension at break (%)	4	400
Density (g/cm^3)	1.25	0.91

However, some P3HB properties, like high crystallinity and low elongation to break, originate films and plastics that are very stiff and brittle, with poor impact strength. In contrast, P(3HB-3HV) co-polymers are less crystalline, have lower melting and glass transition temperatures and as a result, they can be more flexible and ductile, improving the material's thermoplastic properties (Chandra and Rustgi, 1998). Besides 3HV monomers, and depending on the organism, other small and medium-chain monomers may also be incorporated in the polymer, like 3-hydroxy-2-methylbutyrate (3H2MB), 3-hydroxy-2-methylvalerate (3H2MV) and 3-hydroxyhexanoate (3HHx). The polymer properties will then vary accordingly to the percentage of these units and therefore, they can be tuned, increasing the co-polymers range of applications (Philip et al., 2007).

Considering that PHAs mechanical properties are so dependent on the constituent monomers, it is important to understand the mechanisms of its synthesis.

1.4 PHA BIOSYNTHESIS

The microbial PHA biosynthesis has been extensively studied and several biosynthetic pathways are reported for PHA production. Pathway I for example (Figure 1.4) starts with the condensation of two acetyl-CoA molecules to form acetoacetyl-CoA in a reaction catalysed by the enzyme β-ketothiolase. Then, acetoacetyl-CoA reductase acts on acetoacetyl-CoA, forming 3-hydroxybutyryl-CoA. In the last step, PHA synthase catalyses the polymerization via esterification of 3-hydroxybutyryl-CoA into P3HB (Philip et al., 2007).

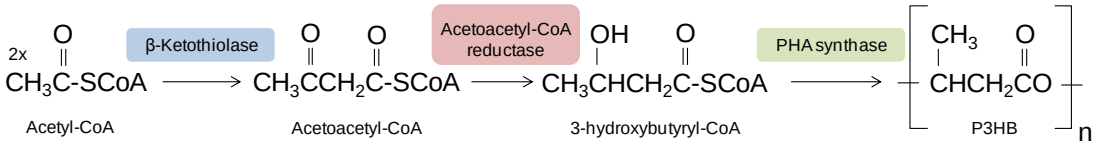

Figure 1.4 – Biosynthetic pathway for the production of P3HB (Braunegg et al., 1998).

There are however other pathways used by some organisms for the production of PHAs. For example, pathway II involves the fatty acid β-oxidation pathway that generates acyl-CoA molecules that can be converted to 3-hydroxyacyl-CoA and then be polymerized by PHA synthase to produce MCL PHA. Subsequent pathways involve the transformation of different molecules derived from other metabolic pathways into 3-,4-,5-,6- hydroxyacyl-CoA units, leading to the formation of alternative co-polymers containing for example, 4-hydroxybutyryl (4HB) monomers (Laycock et al., 2013).

Regardless of whether the PHA producing organism presents all or just some of these pathways, PHA production is subject to internal regulation and can therefore be promoted through activation of specific enzymes involved in PHA synthesis and/or through the inhibition of metabolic pathways that compete for PHA precursors. Consequently, many strategies and different microbial systems can be used to maximize PHA production.

1.5 PHA PRODUCTION IN PURE CULTURE SYSTEMS

In the last decades, PHA production in pure culture systems experienced an extraordinary development with researchers utilizing new substrates, discovering new organisms or even genetically altering existing ones, thus achieving impressive results in terms of PHA productivity, PHA content and diversity of PHA monomer composition (Khanna and Srivastava, 2005; Ienczak et al., 2013).

Several bacteria have long been studied and used for PHA production and the culture conditions for PHA synthesis are carefully adjusted according to the selected organism. Some bacteria have a non-growth-associated PHA production (e.g. *Cupriavidus necator*) and they require the limitation of an essential nutrient like nitrogen or phosphorous, for the synthesis of PHA from an excess carbon source. Cultures of these bacteria usually occur in a two-stage batch production process, where in the first stage cells are grown until a desired concentration (growth phase) followed by a second stage where an essential nutrient is kept limited to allow efficient PHA accumulation (production phase) (Ienczak et al., 2013).

Other bacteria can continuously produce PHA while growing, i.e. they have growth-associated PHA production, and do not require any nutrient limitation. *Alcaligenes latus* and *Burkholderia sacchari* are examples of such bacteria, which are joined by numerous strains of recombinant bacteria (e.g. *E. coli*). In cultivations of these bacteria an optimal ratio between the carbon source and other nutrients is essential to simultaneously enhance cell growth and PHA accumulation in a balanced way (Khanna and Srivastava, 2005).

In all of these pure culture systems, sugars are usually used as the carbon source (e.g. glucose, sucrose, fructose) and typically P3HB is the resultant accumulated polymer. In the case of this polymer, high productivities have been reported with 4.0 g P3HB/L h being produced with sucrose-fed *Alcaligenes latus* (Yamane et al., 1996) and high contents being attained, up to 80%, in glucose fed *Cupriavidus necator* (Ryu et al., 1997). However, as previously referred, PHA co-polymers present better mechanical properties and are therefore preferred in relation to pure P3HB. In general, the production of PHA co-polymers requires media supplementation with substrates like propionic acid for the production of HV monomers (Lee et al., 2008) or γ-butyrolactone for 4HB monomer production (Cavalheiro et al., 2012).

Currently, several PHA plastics produced from pure cultures are already commercialized: Biopol™ (P(3HB-co-3HV)), Nodax™ (P(3HB-co-3HHx)), Enmat™ (P(3HB-co-3HV) and Sogreen™ (P(3HB-co-4HB). They are sold with a price four times higher than conventional plastics, around $5/Kg (Digregorio, 2009), while the cost of polyolefins like polyethylene and polypropylene is situated at $1.3/kg (Platts, 2013). The public may be willing to pay more for products that are considered environmentally friendly, but only to a certain extent. Therefore, PHA prices must decrease in order to promote their marketability, a feature that can only be achieved if production costs decrease.

Considering that the chemically defined media used in pure systems can account by up to 50% of the total production process costs (Choi and Lee, 1997), researchers are now focusing in the utilization of cheaper substrates, like whey (Koller et al., 2008), palm and soybean oil (Singh and Mallick, 2009), biodiesel waste glycerol (Cavalheiro et al., 2012) and lignocellulosic hydrolysates (Cesário et al., 2013). However, the obtained PHA content and productivity is typically lower than those obtained using purified carbon substrates (Khanna and Srivastava, 2005). Also, supplementation of expensive vitamins and co-factors may sometimes be required for polymer production, and due to the 'pure' nature of these systems, media and equipment must be sterilized and operated under strict control. All of these factors contribute to an increase in production costs, but they may be overcome if open mixed cultures are used for PHA production.

1.6 PHA PRODUCTION WITH MIXED MICROBIAL CULTURES

The utilization of mixed microbial cultures (MMCs) for PHA production has been proposed as a means of decreasing PHA production costs due to the possibility of operation under open conditions (no sterility requirements) and utilization of cheap complex feedstocks. Indeed, life cycle assessment and financial analysis have indicated that MMCs PHA production can be financially attractive in comparison to pure culture PHA production (Gurieff and Lant, 2007). But besides the economical perspectives, PHA production with MMCs can positively contribute to the reduction of our environmental footprint, because it presents the double benefit of enabling the production of biodegradable plastics while treating waste streams. Indeed, MMCs can use complex agro-industrial wastes as feedstock for PHA production. Unlike pure culture bacteria that typically use carbohydrates as carbon source, volatile fatty acids (VFAs) (e.g. acetate, propionate and butyrate) are the best precursors for PHA production in MMCs, and they can be easily obtained by fermentation of the waste stream (Reis et al., 2011).

But before the actual PHA production takes place, the MMC commonly undergoes a microbial selection in order to enrich the culture in PHA accumulating bacteria. This is carried out by subjecting the culture to transient feeding conditions in a strategy designated as Feast and Famine (FF). This consists in alternating periods with excess of external carbon (Feast), where organisms take up the carbon and accumulate it as PHA, with periods with no substrate addition (Famine), where only the organisms that accumulated PHA will have carbon reserves and will thus be able to grow. Repeated FF cycles will favor the cell growth on storage products, thus creating a selection pressure for organisms with high PHA-storing capacity. It must be referred however, that in addition to alternating the two phases, studies have shown the importance of imposing long famine phases and short feast phases, i.e. low feast to famine ratio (F/F) values (Dionisi et al., 2006; Albuquerque et al., 2010). It seems that during a long famine phase, there may be a decrease in the cellular synthesis of growth enzymes that leads to an internal growth limitation. As a result, during the following feast phase, cells are not able to grow immediately after substrate supply but are capable to store VFA as PHA, since the latter metabolism does not require physiological adaptation. Bacteria that can physiologically adapt and impose this internal growth limitation will accumulate high PHA contents and will be able to survive during the long famine phase. This physiological adaptation is the key factor that enables the selection of PHA accumulating bacteria with the FF strategy.

The PHA production systems with MMCs can then be described as a process that commonly comprises three-steps (Figure 1.5): (1) waste water or agro-industrial waste acidogenic fermentation to VFAs, (2) selection of a PHA accumulating culture under FF conditions, (3)

PHA accumulation in a batch reactor using sludge from the selected culture fed with the fermented effluent (Albuquerque et al., 2007).

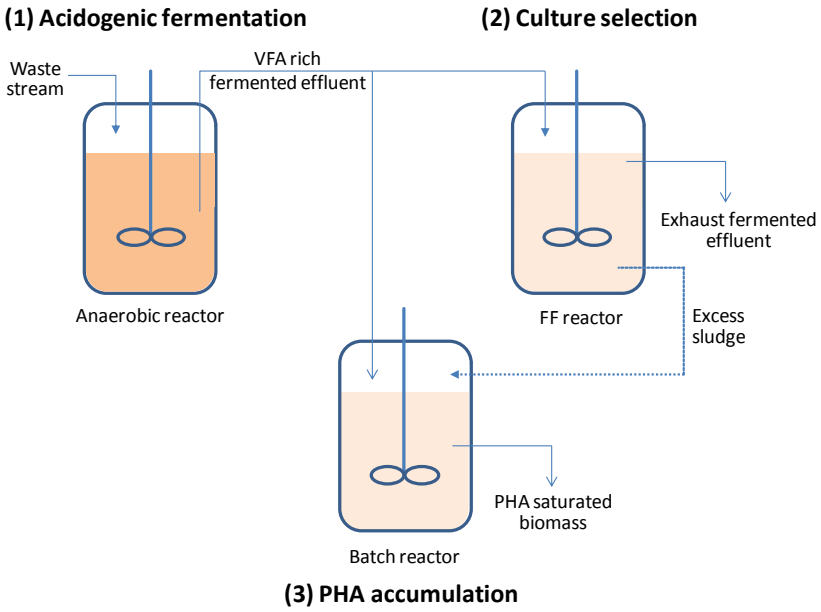

Figure 1.5 – Three-step PHA production process with MMCs using waste streams as feedstock (adapted from Albuquerque et al. 2007)

During the selection step, different approaches of the FF strategy have been used to select distinct PHA accumulating organisms. When carried out completely under aerobic conditions, the process is referred to as aerobic dynamic feeding (ADF), where aerobic bacteria are selected (Figure 1.6 A). When the feast phase is conducted in anaerobiosis, the process consists of repeated cycles of anaerobic feast/aerobic famine that can lead to the selection of polyphosphate and glycogen accumulating organisms (PAOs and GAOs) (Figure 1.6 B).

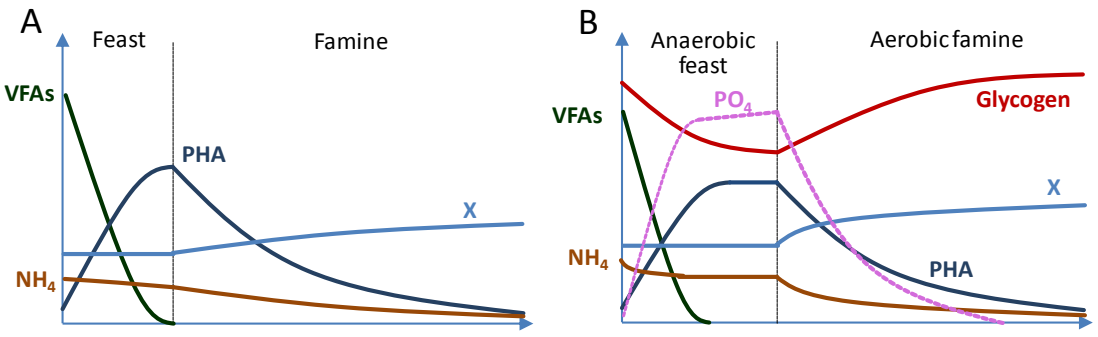

Figure 1.6 – Cycle profile of MMCs selected under a FF strategy. A – Aerobic dynamic feeding with substrate consumption and PHA accumulation during the feast phase, and $NH4^+$ and PHA consumption for growth during the famine phase. B –Anaerobic/aerobic alternating conditions with polyphosphate being used by PAOs (dotted line indicates the phosphate release to the exterior) and glycogen being used by both GAOs and PAOs in the anaerobic feast phase as energy source for substrate consumption and PHA accumulation. In the aerobic famine phase, $NH4^+$ and PHA are consumed for growth and glycogen is replenished from PHA. PAOs take up external phosphate and re-store it as polyphosphate.

In the particular case of PAOs and GAOs, these organisms can accumulate other internal polymers that are used as an energy source for external carbon uptake during the anaerobic periods, where cells cannot use oxidative phosphorylation for ATP production. PAOs can use both glycogen and polyphosphate while GAOs can only use glycogen. These organisms have been extensively studied due to PAOs applicability for phosphorous removal in wastewater treatment (in the so called enhanced biological phosphorus removal (EBPR) systems), while GAOs are studied because they grow in EBPR systems and compete with PAOs for carbon and nutrients, but they do not contribute to phosphorous removal (Oehmen et al., 2006). More recently, GAOs potential for PHA production was thoroughly examined in several works using VFA containing defined media (Dai et al., 2007; Bengtsson, 2009) but also, using agro-industrial wastes, like paper mill wastewater (Bengtsson et al., 2008) and sugar cane molasses (Pisco et al., 2009; Bengtsson et al., 2010). In this last case, up to 37% PHA content was achieved with varied monomer composition that included SCL monomers (3HB, 3HV, 3H2MB, 3H2MV) and MCL monomers (3HHx). These PHA copolymers can be achieved by GAOs even when acetate is fed as the sole carbon source, unlike in the ADF process. This is because a portion of their glycogen is diverted to propionyl-CoA during the generation of ATP for VFA uptake, in order to balance their internal cell levels of NADH.

In the case of aerobic MMCs selected in ADF processes, P(3HB) accumulation levels up to 89% have thus far been achieved with an acetate-fed culture (Johnson et al., 2009). This is a PHA content comparable to the best accumulation values reported in pure cultures. But the utilization of fermented agro-industrial wastes as feedstock have also led to very interesting PHA accumulation values with 75% PHA content using sugar cane molasses (Albuquerque et al., 2010) and 77% using paper mill wastewater (Jiang et al., 2012). Moreover, the accumulated PHAs were composed of P(3HB-3HV) co-polymers with a 3HV content of 39% and 14% (Cmol base), respectively. Currently, many other fermented agro-industrial wastes have been successfully tested as potential feedstock for PHA production, like olive oil mill pomace (Waller et al., 2012), olive oil mill effluent (Dionisi et al., 2005; Beccari et al., 2009), waste activated sludge (Morgan-Sagastume et al., 2010), palm oil mill effluent (Mumtaz et al., 2010) and mixtures of sludge and food waste (Chen et al., 2013). The resultant PHAs were also composed of co-polymers that displayed a diverse monomeric composition.

This diversity in the final polymer composition is dependent on several interlinked factors, like the type of feedstock used, the selected microbial species and the operational conditions. During the feedstock fermentation, different VFAs can be produced with variable relative proportions, and it has been found that the substrate feeding composition can strongly affect the PHA monomer diversity (Serafim et al., 2008; Albuquerque et al., 2011). Typically, organic acids with an even carbon number (e.g. acetate and butyrate) lead to P3HB formation, while odd

numbered VFAs (e.g. propionate and valerate) allow the production of 3HV monomers, since a greater number of propionyl-CoA precursors are formed in the latter case. So, by tuning the substrate VFA composition, it is possible to tailor the produced PHA into the desirable co-polymer. However, this possibility is also dependent on the microbial species present in the MMC (Carvalho et al., 2013). As previously referred, different bacteria present specific metabolic pathways for PHA production, so the substrate conversion into different precursors is species-dependent and consequently, so is the production of different PHA monomers.

The selection of different microbial species is also dependent on the substrate composition and on operational parameters (Jiang et al., 2011a, 2011b). Several studies using different feedstocks and operational conditions (synthetic and real) obtained MMCs with diverse microbial communities. *Amaricoccus*, *Thauera*, *Azoarcus*, *Paracoccus*, *Zoogloea*, *Comamonas*, are examples of the variety of bacteria genus and *Plasticicumulans acidivorans* an example of a bacterial species, that can be found/selected in PHA accumulating aerobic MMCs (Dionisi et al., 2006; Lemos et al., 2008; Beccari et al., 2009; Villano et al., 2010; Jiang et al., 2011a). Recently, insight on these organisms' substrate preferences and the resultant PHA that is produced has been reported (Jiang et al., 2011b; Albuquerque et al., 2013; Carvalho et al., 2013; Marang et al., 2013) providing important information that may be used for manipulation of the microbial community structure and consequently lead to optimization of the PHA production.

Finally, it is important to refer that the system's operational conditions can also be adjusted in order to tune the polymer properties. Thus far, temperature (Jiang et al., 2011a), pH (Villano et al., 2010; Chen et al., 2013), feeding regime (Albuquerque et al., 2011; Chen et al., 2013) organic loading rate (Dionisi et al., 2006; Carvalho et al., 2013), sludge retention time (Johnson et al., 2009) and feast to famine ratio (Albuquerque et al., 2010) have all been shown to influence the PHA content and composition, whether directly, or indirectly, through its influence on the microbial species selection of the culture.

All of these studies that have been conducted thus far have led to a greater awareness of the potential of MMCs as PHA producers and have revealed important information for the design of strategies and systems that can maximize PHA production. Research in this field is continuously occurring and new feedstocks and new operating conditions are tested in order to bring forward this sustainable polymer production process. In spite of the researchers' dedicated efforts, PHA production with MMCs has been mainly restricted to the utilization of aerobic organisms, while in fact, the diversity of bacterial species that can produce and accumulate PHA is much wider. Other organisms present alternative means of producing energy besides oxidative phosphorylation, and they can also make use of PHA in order to thrive in their natural habitat. An example of such organisms, are the photosynthetic bacteria.

1.7 PHA PRODUCTION IN PHOTOSYNTHETIC BACTERIA

Photosynthesis is one of the first and oldest processes that organisms have used for energy production. It was this process that millions of years ago transformed the Earth's reductive atmosphere (CO_2, H_2, H_2S) into the oxidative atmosphere that we know today, and that influenced evolution by giving rise to the development of a whole new microbial diversity of aerobic organisms.

Photosynthesis consists in converting light energy into a usable form of chemical energy and is a process that can be used by a great diversity of organisms, both eukaryotic and prokaryotic. For simplicity, the photosynthetic microorganisms can be divided into four groups: microalgae, cyanobacteria, green bacteria and purple bacteria. The first two groups perform an oxygenic photosynthesis (i.e. oxygen is produced) in a process that occurs in two distinct stages. The first stage includes light dependent reactions and takes place in specialized structures (chloroplasts) where light energy is absorbed, driving the synthesis of ATP and NADPH in a process coupled to the formation of oxygen from water (Figure 1.7). The second stage includes the dark reactions (independent of light) where the produced ATP and NADPH are used for CO_2 fixation into carbohydrates through the Calvin cycle.

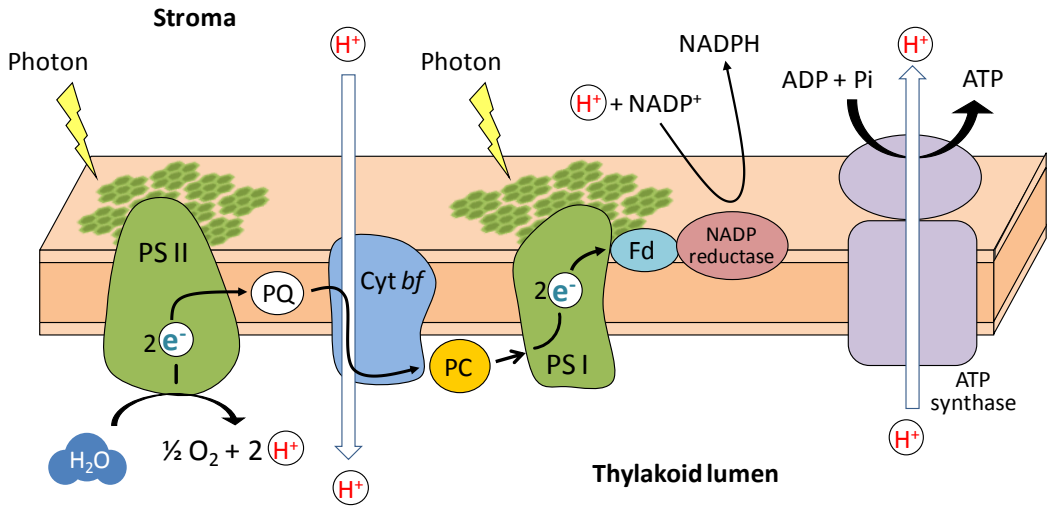

Figure 1.7 – Light reactions of photosynthesis occurring in the chloroplast of microalgae and cyanobacteria (Cooper, 2000; Prescott et al., 1999). Photons are absorbed in the pigments associated to photosystems I and II (PS I and PS II) and the energy derived from the photon absorption is used in PS II to split water to oxygen. Electrons from the water are carried by plastoquinones (PQ) into the cytochrome *bf* complex, and their transference to a lower energy state is coupled with protons pumping to the thylakoid lumen. Electrons are then transferred to PS I by plastocyanin (PC), where photon absorption again generates high energy electrons that are transferred to ferrodoxin (FD) and used to reduce $NADP^+$ to NADPH in the stroma. The energy stored in the proton gradient created during the electron transport is used by ATP synthase to convert ADP to ATP.

In the case of green bacteria and purple bacteria, these organisms perform an anoxygenic photosynthesis (Figure 1.8). Photosystem II is absent and therefore they cannot use water and evolve oxygen. They only have photosystem I and they use it for ATP synthesis. Because this occurs with a cyclic electron flow, no NADPH is synthesized. Instead, these bacteria use electron donors like hydrogen, hydrogen sulfide and organic molecules to regenerate NADH and NADPH. Then, they can use the produced ATP and NADPH to fixate CO_2 or, depending on the external conditions, they may use ATP to take up external organic molecules as carbon source (heterotrophic metabolism).

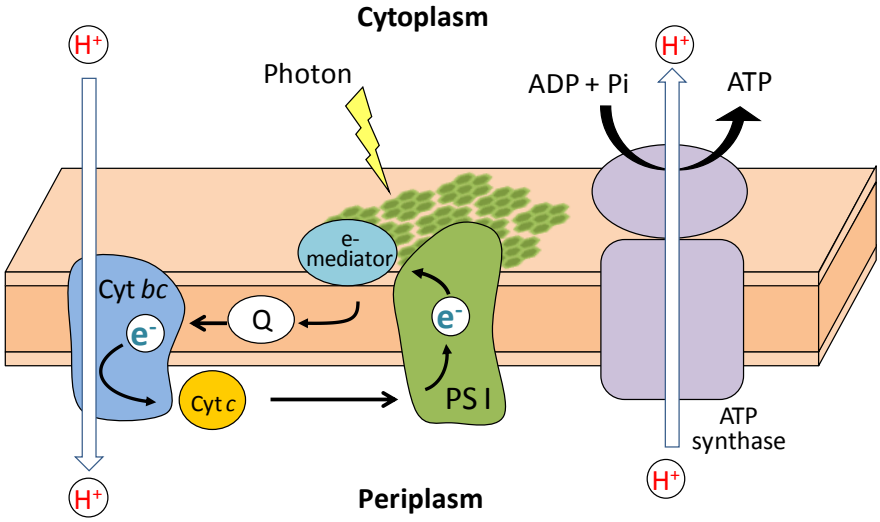

Figure 1.8 – Tentative scheme of light reactions of photosynthesis occurring in the chlorosomes of green bacteria and in membrane systems of purple bacteria (Cooper, 2000; Prescott et al., 1999). In these organisms, photosynthesis occurs in a cyclic electron flow pathway. High energy electrons are generated by photon absorption in PS I and are transferred to the cytochrome *bc* complex via an electron mediator and a quinone (Q). At the cytochrome *bc* complex, electrons are transferred to a lower energy state and protons are pumped to the periplasm. The electrons then return to PS I, carried by cytochrome *c*. The energy stored in the proton gradient created during the cyclic electron flow is used by ATP synthase to convert ADP to ATP.

Another important aspect of green and purple bacteria, is that they possess bacteriochlorophylls (*a* or *b* for purple bacteria, *a* or *c*, *d*, *e* for green bacteria), which are photosynthetic pigments slightly different than the chlorophylls of microalgae and cyanobacteria. Though the two types of pigments share an absorption spectra around 350 to 500 nm, bacteriochlorophylls can also absorb longer wavelengths, far-red light, around 850-910 in the case of bacteriochlorophyll *a* and 1020-1035 in the case of bacteriochlorophyll *b*. This gives green and purple bacteria the possibility of using other wavelength ranges when competing with microalgae and cyanobacteria for light energy. The four groups share, however, a common photosynthetic pigment, carotenoides (e.g. β-caroten), that have an absorption range around 400-600 nm, in the middle of the visible light range (Prescott et al, 1999).

From the four mentioned groups, PHA production only occurs among the prokaryotic organisms. Microalgae do not accumulate PHA (thus far no observation has been reported in Eukaryotes (Shively, 2006)), but it is also important to consider these organisms, because they are easily adaptable to different environments and therefore, they are frequently present in photosynthetic mixed cultures.

The preferred mode of growth of microalgae is photoautrotophy, but they are also capable of photoheterotrophy, taking up organic molecules like glucose, acetate, glycerol and ethanol as carbon source (Chen and Johns, 1996; Muñoz and Guieysse, 2006; Perez-Garcia et al., 2011). For this reason, several works have discussed the microalgae potential for wastewater treatment with bacterial-algae consortiums (Ogbonna et al., 2000; Safonova et al., 2004; Perez-Garcia et al., 2010). Microalgae have also been thoroughly studied due to their hydrogen production capacity (Ghirardi et al., 2000; Rashid et al., 2011), and though they do not accumulate PHA, they can produce and accumulate other commercially attractive molecules like antioxidants, carotenoides for β-carotene production and lipids for biofuel production (Iersel, 2009).

As previously referred, photosynthetic PHA production has only been reported in organisms of cyanobacteria, green bacteria and purple bacteria. A brief description will be given forthwith.

PHA production in cyanobacteria has drawn great attention in recent years due to their ability to accumulate PHB under photoautothrophic conditions with just some requirements of simple inorganic nutrients (e.g. phosphate, nitrate) and micronutrients (Balaji et al., 2013). Thus far, several PHA accumulating cyanobacteria species have been identified (e.g. *Anabaena* spp., *Spirulina* spp., *Synechococcus* spp., *Synechocystis* spp., *Nostoc* spp. and *Oscillatoria* spp.) (Miyake et al., 1996; Wu et al., 2001; Balaji et al., 2013) but typically, their PHA content is quite low, around 2 - 6 % of cell dry weight (cdw) (Bhati et al., 2010). Higher PHA contents have been reported (40-45% dcw) but they were only achieved by multi-stage cultivation processes with additives supplementation (Wang et al., 2013). Currently, metabolic engineering is being used to improve cyanobacteria accumulation capacity, monomer diversity and utilization of alternative substrates (Balaji et al., 2013; Wang et al., 2013). Considering this last case of substrate alternatives for cyanobacteria, though cyanobacteria are unique in their ability of utilizing CO_2 for PHA production, with great potential for CO_2 recovery during off-gas streams treatment, they are very limited when utilizing organic molecules as carbon source, presenting a slow growth under heterotrophy (Smith et al., 1982). This restricts their substrate utilization range, hampering the utilization of wastewaters and solid wastes as feedstock. However, this is a recent field of research and the CO_2 fixation capacity of cyanobacteria seems to be an interesting characteristic that may lead to the development of sustainable PHA production process with CO_2 being captured for the production of biodegradable biopolymers.

In the case of the green bacteria, PHA production has, thus far, been only reported for the genus *Chloroflexus* (Pierson and Castenholz, 1974; Fuller, 1995; Sirevåg, 1995). Besides autotrophy, *Chloroflexus* are capable of utilizing several organic compounds like acetate, lactate, butyrate, alcohols, and sugars for growth under anaerobic illuminated conditions and in aerobic dark conditions. Though these organisms present a versatile metabolism, not much research has been done to explore the potential of these organisms for PHA production. Possibly this is due to the fact that *Chloroflexus* present an optimal growth at high pH and temperatures between 50 – 60 º C, thus being commonly found in alkali hot springs, which is their natural habitat (Sirevåg et al., 1995).

The most well studied PHA accumulating photosynthetic microorganisms are the purple bacteria. This group contains some of the most versatile bacteria that exist, being capable of growing under phototrophic conditions in the light and under chemotrophic conditions in the dark, either autotrophically (fixating CO_2) or heterotrophically using oxygen or inorganic molecules as terminal electron acceptors in respiratory processes (Imhoff, 2006a). Though they have so many possible metabolic combinations, they still have a preferred mode of growth. Consequently, these organisms can be divided in two groups: the purple sulfur bacteria (γ-proteobacteria) and the purple non-sulfur bacteria, which are mostly α-proteobacteria with the exception of one genus that is β-proteobacteria (Prescott et al., 1999).

Purple sulfur bacteria typically grow photosynthetically under anaerobic conditions using hydrogen, sulfide and sulfur as electron donors, producing sulfur granules that can be deposited internally or externally, depending on the organism family. Several species like *Chromatium* spp., *Lamprocystis* spp., *Amoebobacter* spp., *Thiosystis* spp., and *Ectothiorhodospira* spp. were found to be capable of accumulating PHA. Particularly, *Thiosystis* spp., *Ectothiorhodospira spp.* and *Chromatium* spp. attained PHB contents of 83 %, 57 % and 58 % cdw, respectively, when fed with acetate under illumination (Liebergesell et al., 1991).

As for the purple non-sulfur bacteria (PNS bacteria), these organisms also typically grow under anaerobic conditions in the light, but they present a much more highly diverse and flexible metabolism in relation to the purple sulfur bacteria. In fact, their preferred mode of growth is photoorganoheterotrophy, using organic molecules both as electron donors and carbon source. Nevertheless, many species can also use hydrogen and reduced sulfur compounds as photosynthetic electron donors. Besides phototrophy, some PNS can grow equally well in the dark, in aerobic conditions or anaerobically, using fermentative processes for energy production. In this latter case, reduced organic compounds are produced (e.g. succinate, lactate, acetate, propionate, formate) as well as CO_2 and H_2. With such a versatile metabolism, PNS bacteria are

widely distributed in nature, being found in lakes, ponds, sediments, moist soils and paddy fields (Imhoff, 2006b).

The most representative and well studied genus of the PNS bacteria are the *Rhodospirillum*, *Rhodobacter* and *Rhodopseudomonas*. Numerous species of these genus have been reported as PHA accumulators, and in a study of Liebergesell et al, 1991, some *Rhodobacter* spp. organisms were capable of accumulating P3HB up to 70% cdw using acetate as carbon source in anaerobic illuminated conditions. Moreover, 3HV monomers production could be observed in organisms from the three refereed genus, when cells were cultivated in the presence of odd-carbon-numbered organic acids (e.g. propionate and valerate). Other works have studied the effect of various carbon and nitrogen substrates on the PHA production of *Rhodobacter sphaeroides* (Khatipov et al., 1998) and even the utilization of an agro-industrial waste (fermented palm oil mill effluent) was also tested as feedstock for PHA production in that same species, being obtained a PHA content around 60% cdw (Hassan et al., 1997).

Despite the fact that PNS have great potential as PHA producers, they have been the subject of intensive research not because of their polymer accumulation capacity, but because of their ability for hydrogen production (Basak and Das, 2006; Kapdan and Kargi, 2006). It must be referred that when PNS bacteria are cultivated in anaerobic illuminated conditions and are fed with lower energetic organic acids like acetate, propionate, malate, succinate and lactate, cells use the photosynthetically produced ATP to take up these organic molecules, but they need an electron acceptor to dissipate the reducing power (e.g. NADH, NADPH) produced during the cells metabolism. PNS bacteria can dissipate this energy by two means: by hydrogen production and by PHA accumulation. In the case of hydrogen, PNS bacteria use their nitrogenase enzyme that catalyses hydrogen production in the absence of molecular nitrogen (Eq. 1).

$$2\ H^+ + 2\ e^- + 4\ ATP \rightarrow H_2 + 4\ ADP + 4\ Pi \qquad \text{(Eq. 1)}$$

Since nitrogenase is an enzyme with the primary function of N_2 fixation for NH_4^+ production, its utilization for reducing power dissipation through hydrogen production is highly dependent on the presence of N_2 and NH_4^+. With N_2 present, the nitrogen fixation reaction dominates. With NH_4^+ present (which is a salt frequently used as a nitrogen source in bacteria cultivation) the nitrogenase synthesis is repressed and its activity is inhibited (Koku et al., 2002). Furthermore, this is a highly energy demanding reaction, so cells must consume large amounts of energy (ATP) in order to re-oxidize reduced molecules like NADH, avoiding their accumulation and maintaining cell homeostasis. Alternatively, if cells produce PHA , no ATP is required and cells can directly re-oxidize the reduced agents by, for example, reducing acetoacetyl-CoA molecules to 3-hydroxybutyryl-CoA, as previously referred in section 1.4. In this case, PHA is not only

accumulated as a carbon reserve, but most importantly, it is used as a reducing power sink. The utilization of one pathway or the other by the cells is then dependent on the amount of ATP available, on the nitrogenase enzyme requirements, but also, on the type of organic acids that are fed (Wu et al., 2012). It is more likely that cells use the PHA synthesis pathway when they are fed with molecules that are easily converted to this polymer, like acetate, propionate and butyrate. In the case of other organic acids, and particularly in the case of the ones from the tricarboxylic acids cycle (TCA) like malate and succinate, it is then more likely that cells use the hydrogen production pathway, since PHA production is more inaccessible (Han et al., 2012). Taking all of this into consideration, PHA production with PNS bacteria seems to have a great potential as long as VFA-rich and ammonium-containing streams are used as feedstocks. Indeed, this is the case for most fermented feedstocks from wastes.

In summary, many different types of photosynthetic bacteria present interesting characteristics for PHA production. *Cyanobacteria* can fixate CO_2 for PHA production, green bacteria *Chloroflexus* are thermophilic PHA producers and purple bacteria are versatile photoheterotrophic bacteria that use VFAs as the best organic molecules for PHA synthesis. The selection of organisms from one group or the other will depend on the type of stream that is to be used/treated and on the chosen operational conditions. Previous studies have not focused on achieving photosynthetic PHA production in MMCs, despite the potential advantages in MMC PHA production over pure cultures that were stated previously.

1.8 MOTIVATION AND THESIS OUTLINE

The growing public concern with traditional plastics and their negative impact on the environment, has led to an increasing demand for biodegradable plastics. Researchers and industries have answered this demand with new biodegradable polymers, and PHA, which is one of the top candidates for the replacement of conventional plastics, is already on the market. However, its current prices are far from competitive, and researchers are putting their efforts into devising new strategies that may lead to a decrease in PHA production costs.

The objective of this Thesis work was precisely to propose a new system for PHA production that has the potential to lower operational costs and decrease the cost of PHA production to more affordable levels. From the introduction that is given in Chapter 1, it can be affirmed that MMC systems have potential to lower costs through facilitating the use of waste feedstocks under non-aseptic conditions. However, the potential for photosynthetic PHA production was not considered in those systems and consequently, it has never been thoroughly tested. Chapter 2 brings some insight on this matter and discloses the first results on the utilization of a

photosynthetic mixed culture for PHA production. This photosynthetic mixed culture (PMC) is proposed as an alternative to the utilization of common MMCs by presenting the advantage of not requiring any aeration, thus potentiating reduced operational costs. Also, the PMC system uses light as its energy source, opening up the possibility of a sunlight driven PHA production system and thus reducing even more the energy costs.

Chapter 3 addresses the impact of alternating the PMC process operation between light and dark periods, since in order to enable the future use of sunlight in this PHA production system, the PMC must be capable of enduring dark periods. Chapter 3 shows the first results from subjecting the PMC to alternating dark/light periods and the potential advantages that can be realized from this change in operation.

Since achieving a sustainable PHA production process is only possible if cheap residues are used as feedstock, Chapter 4 shows the results obtained from testing the capacity of a PMC to use substrates that are commonly found in fermented agro-industrial residues. The tests were conducted with synthetic substrates for a better understanding of the synergistic effects between them, creating a base of knowledge for future utilization of real feedstocks.

The PMCs studied in Chapters 2, 3 and 4, were all selected and operated using the feast and famine regime, adopting the strategy that has been commonly applied for the selection of PHA accumulating bacteria. However, a completely new approach for selecting PHA accumulating photosynthetic bacteria was tested in Chapter 5. A permanent feast regime was used and bacteria were no longer selected due to their ability to grow on stored PHA, but due to their unique ability of using PHA as an electron sink.

Finally, a summary of the most important results and major conclusions of this thesis are provided in Chapter 6, along with suggestions for future research work.

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2

Polyhydroxyalkanoates production by a mixed photosynthetic consortium of bacteria and algae

Summary *For the first time, a mixed photosynthetic culture (PMC) consisting of a consortium of bacteria and algae was investigated for its capacity to accumulate polyhydroxyalkanoates (PHA). The culture was subjected to a feast and famine regime in an illuminated environment without supplying oxygen or any other electron acceptor. The PMC accumulated PHA during the feast phase and consumed it in the famine phase, where the PHA consumption was made possible due to oxygen production by algae. The internal cycling of carbohydrates was also observed, which was likely linked to bacterial glycogen being used as an additional source of energy for acetate uptake during the feast phase, and restored in the famine phase via PHA degradation. The PMC reached a PHA content of 20%, with a PHA storage yield per acetate similar to aerobic systems, opening up the possibility of a new sunlight-driven PHA production process without the need for aeration.*

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2.1 INTRODUCTION

Polyhydroxyalkanoates (PHA) are biopolymers synthesized by numerous bacterial species as intracellular carbon and energy reserves. These polyesters have chemical and physical properties similar to conventional plastics, which added to their biodegradability and biocompatibility, makes them a promising green alternative to petrochemically based plastics (Braunegg et al., 1998; Verlinden et al., 2007).

Industrial PHA production is already a reality, but the fact that it is based on the utilization of pure microbial cultures results in high PHA production costs, where the final product is significantly more expensive than traditional synthetic plastics (Reis et al., 2003; Gurieff and Lant, 2007). Efforts to create more economical PHA production strategies have surged in recent years with the utilization of mixed microbial cultures (MMC) (Dionisi et al., 2004; Reis et al., 2011; Mohan and Reddy 2013). The application of MMC avoids the cost of equipment sterilization and the use of expensive refined substrates, as they can be operated in open systems and utilize cheap industrial fermentable by-products (Reis et al., 2011).

Operational strategies involving the feast and famine (FF) regime have been applied in order to select for MMC with high PHA storage capacities. The FF regime involves intermittent substrate feeding where the external carbon is taken up and accumulated intracellularly as PHA, followed by phases without substrate addition that favor cell growth on storage products, thus creating a selection pressure for organisms capable of storing PHA (Reis et al., 2003).

This FF strategy has been successfully applied for fully aerobic systems (Dionisi et al., 2004; Johnson et al., 2009; Albuquerque et al., 2010) and for systems with alternating anaerobic/aerobic cycles (Dai et al., 2007; Bengtsson, 2009; Pisco et al., 2009). PHA accumulation to levels as high as 75% in aerobic systems (Albuquerque et al., 2010) and 37% in anaerobic/aerobic systems (Bengtsson et al., 2010), have already been achieved when fed with volatile fatty acids (VFA) from fermented industrial by-products (e.g. sugarcane molasses). In each of these aforementioned cases, an electron acceptor (e.g. oxygen) must be provided, usually through aeration. Aeration is widely recognized as the most energy intensive requirement of mixed culture bioreactors, such as those applied for activated sludge wastewater treatment plants (Rosso et al., 2008). The need for aeration substantially increases the operational costs of the MMC process for PHA production.

One of the goals of this work was to enrich a MMC capable of accumulating PHA without supplying any aeration. In order for this to occur, cells must have an alternative source of energy production, and a means of oxidizing reduced molecules (e.g. NADH, NADPH), avoiding their accumulation and maintaining cell homeostasis. Most cells produce energy in the form of ATP

by oxidative phosphorylation - a pathway that cells utilize only when electron acceptors such as oxygen are present. Light can be used as an alternative energy source, where photosynthetic bacteria harvest light by their photosystems, producing the ATP needed to take up external carbon sources. Furthermore, cells consume reducing equivalents such as NADH during the storage of carbon as PHA. PHA accumulation has been observed within pure cultures of the photosynthetic purple bacteria, in both sulfur and non-sulfur bacteria (Liebergesell et al., 1991). Nevertheless, photosynthetic mixed culture systems have never before been operated for PHA production, despite the potential advantages of MMCs as compared to pure cultures.

In order to successfully achieve an enrichment of photosynthetic PHA producing organisms, it was hypothesized that a FF regime would be the most appropriate, similarly to aerobic MMCs. However, PHA consumption in the famine phase leads to reducing equivalents that must be oxidized. PHA oxidation can be achieved in illuminated environments (without external supply of electron acceptors) through the growth of algae, which produce oxygen photosynthetically. This oxygen can then be used as electron acceptor by PHA storing bacteria, enabling PHA consumption during the famine phase.

In this work, a mixed photosynthetic culture (PMC) was operated in an illuminated and non-aerated environment using a FF regime to enrich for PHA accumulating organisms. The aim of this study was to develop a consortium of bacteria and algae, where bacteria accumulate PHA during the feast phase, and consume it in the famine phase using the oxygen produced by algae. The capability of the PMC to accumulate PHA was studied, as well as the impact of illumination and aeration on its metabolic behavior.

2.2 MATERIALS AND METHODS

2.2.1 Photosynthetic mixed culture enrichment

The PMC studied in this work was inoculated with sediments from a local pond and operated for over 1 year in a 4.4 L sequencing batch reactor (SBR). The SBR was illuminated internally with a halogen lamp (200 W) at a light intensity of 150 W/m^2, which corresponds to a volumetric intensity of 1.3 W/L of culture broth. The SBR was operated in 8h cycles with a hydraulic retention time (HRT) and sludge retention time (SRT) of 6 days. Temperature was controlled at 30ºC and argon was continuously sparged (10mL/min) to prevent surface aeration. pH was controlled at 6.5 using 0.5 M HCl. During each cycle, the SBR was fed with equal amounts of culture medium (containing per liter 0.8 g $MgSO_4.7H_2O$, 1.6 g NaCl, 1.1 g NH_4Cl, 0.2 g $CaCl_2.2H_2O$, 8.2 g NaAcetate.$3H_2O$, 20 mL iron citrate solution (1.0g/L), 4 mL trace

element solution) and phosphate medium (containing per liter 0.13 g KH_2PO_4 and 0.17 g K_2HPO_4).

2.2.2 Batch tests under different illumination and aeration conditions

In order to evaluate the effect of illumination and aeration on PMC response, batch tests were performed using the SBR sludge in a separate 500 mL reactor operated under the same conditions of temperature, illumination and pH control. During each test, a proportionally equivalent quantity of phosphate and culture medium was added to 350 mL of culture broth collected from the SBR at the end of an 8h cycle.

In tests applying illumination without aeration (henceforth referred to as Light-Argon conditions), the batch reactor was externally illuminated with a light intensity equivalent to the SBR on a volumetric basis, and argon was continuously sparged. Tests without illumination or aeration (henceforth referred to as Dark-Argon conditions), involved argon sparging, and the reactor was covered to ensure no light penetration. The reactor was also covered in tests without illumination or aeration (henceforth referred to as Dark-Aerated conditions), where argon sparging was replaced by continuous aeration (~ 90% oxygen saturation). Replicate tests were performed as indicated in Table 1.

2.2.3 PHA accumulation tests

Two batch tests were performed to assess the PHA accumulation capacity of the PMC. The initial acetate concentration in the first test was 15 C-mmol/L, 5 times higher than the SBR, where the culture medium and phosphate medium were fed in the same proportion as in 2.2.2. In the second test, 350 mL of culture broth was first settled and 200 mL of the supernatant was discarded in order to remove most of the $NH4^+$. Then, 30 mL of phosphate medium was added as well as 190 mL of culture medium without acetate and $NH4^+$. Pulses of an acetate solution (2.5 C-mol/L) were added throughout the test, preventing carbon depletion and substrate inhibition. In both tests, Light-Argon conditions were used as described in 2.2.2.

2.2.4 Analytical Methods

The acetate concentration was determined by high-performance liquid chromatography (HPLC) using an IR detector and a BioRad Aminex HPX-87H column. 0.01 N sulfuric acid was used as eluent, with an elution rate of 0.6 mL/min and a 50ºC operating temperature.

Total carbohydrates were determined using the method described by Lanham et al. (2012), with minor modifications. Briefly, the pellet of centrifuged samples was washed twice with 0.9 % NaCl and lyophilized overnight. The biomass was then mixed with 2 mL of 0.6 M HCl and digested for 2h at 100ºC. The supernatant was filtered (0.2 µm membrane) and glucose was analyzed by HPLC as described above, using an elution rate of 0.5 mL/min and a 30ºC operating temperature.

PHA determination was performed by gas chromatography according to Serafim et al. (2004), with minor modifications. Lyophilized biomass was digested for 2h at 100ºC with 1 mL of chloroform and 1 mL of methanol with 3% sulfuric acid. The organic phase was extracted and injected into a gas chromatograph coupled to a Flame Ionization Detector (GC-FID Varian CP-3800) and equipped with a ZBWax-Plus column. Helium was the carrier gas at a flow rate of 1 mL/min and heptadecane was used as the internal standard during gas chromatography.

Ammonia was analyzed using an ammonia gas sensing combination electrode ThermoOrion 9512. Volatile suspended solids (VSS) were determined according to Standard Methods (APHA, 1995).

The light intensity was measured using a Li-COR light meter LI-250 A equipped with a pyranometer sensor LI-200 SA.

2.2.5 Microbial characterization of the PMC

Biomass samples were examined microscopically for morphological observation, visualization of intracellular PHA granules (through Nile blue staining) and for bacterial community analysis by fluorescence *in situ* hybridization (FISH). Nile blue staining was performed on wet biomass according to Bengtsson et al. (2008). For FISH analysis, sludge samples were fixed with paraformaldehyde or ethanol as described by Nielsen et al. (2009). The following specific oligonucleotide probes were employed: ALF969 for *Alphaproteobacteria* (Oehmen et al., 2006), BET42a for *Betaproteobacteria*, GAM42a for *Gammaproteobacteria*, Delta495a for *Delta-Proteobacteria*, CF319a for *Cytophaga-Flavobacteria* and HGC69a for High GC content Gram-positive bacteria (*Actinobacteria*). To scan the purple non sulfur Bacteria group (PNS) the following probes were also employed: within the *Alphaproteobacteria*, GRb was used for *Rhodobacter* and *Roseobacter*, Rhodo-2 for *Rhodospirillum* and Rhodopseud for *Rhodopseudomonas*; within the *Betaproteobacteria* RHC439 was used for *Rhodocyclus*. To scan known PHA accumulating bacteria, the following probes were employed: PAR651 for *Paracoccus*, Azo644 for *Azoarcus*, Thau832 for *Thauera*, ZRA23a for *Zoogloea* and AMAR839 for *Amaricoccus*. To scan glycogen accumulating organisms, the probe set GAOmix

(GB G2 + GAOQ989) was employed for *Competibacter phosphatis*, TFOmix (TFO_DF218 + TFO_DF616) for *Defluviicoccus vanus* – cluster 1, DFmix (DF988 + DF1020) for *Defluviicoccus vanus* – cluster 2, DF198 for *Defluviicoccus vanus* – cluster 3, DF181A + DF181B for *Defluviicoccus vanus* – cluster 4. Finally, PAOmix (PAO462 + PAO651 + PAO846) was employed for *Accumulibacter phosphatis*. Each specific probe was applied with a Cy-3 label, together with a FITC-labelled EUBMIX probe for all *Bacteria* (EUB338 and EUB338-II and III). Details on oligonucleotide probes are available at probeBase (Loy et al., 2007) unless otherwise indicated. An Olympus BX51 epifluorescence microscope was used for the microscopic observations of biomass samples.

2.2.6 Calculation of kinetic and stoichiometric parameters

The biomass PHA content was calculated as a percentage of VSS on a mass basis ($\%PHA = 100 \times g\ PHA/\ g\ VSS$), where VSS includes active biomass (X), PHA and total carbohydrates. Active biomass was calculated by subtracting PHA and total carbohydrates from VSS.

The maximum specific substrate uptake rate (-q_S in Cmol Acet/Cmol X h), maximum specific PHA production rate (q_P in Cmol PHA/Cmol X h) and maximum specific carbohydrate utilization rate (q_{Carbs} in Cmol Carbs/Cmol X h) were determined by adjusting a linear regression line to the experimental concentrations determined over time and dividing the slope of the fitting at time zero by the concentration of active biomass at that point.

The yields of PHA ($Y_{PHA/S}$ in Cmol PHA/Cmol Acet) and carbohydrates ($Y_{Carbs/S}$ in Cmol Carbs/Cmol Acet) per substrate consumed were calculated by dividing the amount of PHA formed or the carbohydrates consumed by the amount of acetate consumed. The yield of carbohydrates per PHA consumed ($Y_{Carbs/PHA}$ in Cmol Carbs/Cmol PHA) was calculated by dividing the amount of carbohydrates formed by the amount of PHA consumed in the famine phase. Similarly, the yield of PHA per carbohydrates consumed ($Y_{PHA/Carbs}$ in Cmol PHA/Cmol Carbs) was determined by dividing the amount of PHA formed by the amount of carbohydrates consumed.

2.3 RESULTS AND DISCUSSION

2.3.1 Mixed photosynthetic culture enrichment

The enrichment of the PMC used in this study was carried out under a FF regime in an illuminated SBR without aeration, and resulted in a mixed population of photosynthetic bacteria and algae. During steady-state operation, the PMC presented a biomass concentration of 1.4 ±

0.1 g/L of VSS (volatile suspended solids) and dissolved oxygen concentrations measured in the SBR were constantly zero. Figure 2.1 shows the evolution of the culture's performance during the enrichment period of 50 days.

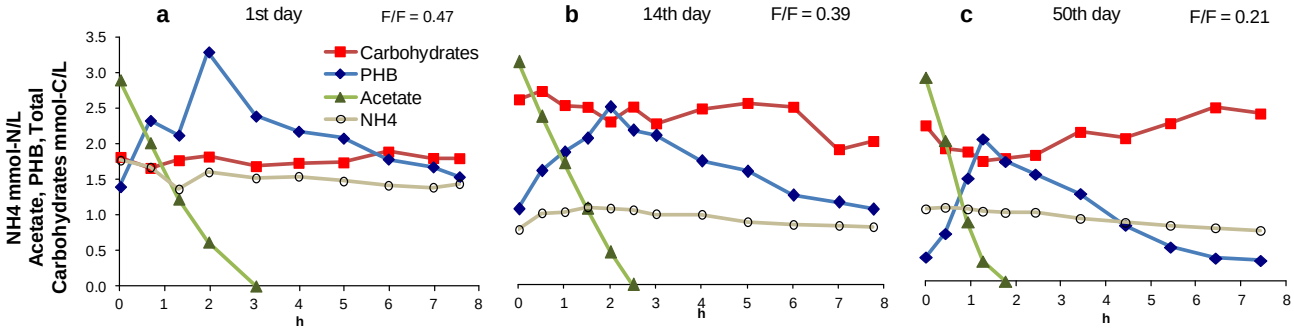

Figure 2.1 – Evolution of the culture's behavior during its enrichment in an 8h cycle SBR subjected to a feast and famine regime with acetate as carbon source under illuminated, non-aerated conditions.

It can be observed that from the beginning of the enrichment period, the PMC was capable of taking up all of the acetate and accumulating it as polyhydroxybutyrate (PHB), a homopolymer of 3-hydroxybutyrate. This polymer was the only form of PHA that was accumulated by the PMC, which was expectable since acetate was used as the sole carbon source. After the depletion of acetate, PHB was consumed during the famine phase. The profile of PHA accumulation and consumption became more accentuated along the enrichment period, suggesting an increase in PHA accumulating bacteria (Figure 2.1a to c). A gradual increase in acetate uptake rate was simultaneously observed, which resulted in shorter feast phases and longer famine phases. The F/F ratio dropped from 0.47 to 0.21 during the enrichment. According to previous studies with aerobic MMCs, 0.21 is in the range of F/F ratios (0.20 - 0.25) that best promote the selection of PHA accumulating organisms (Dionisi et al., 2006; Albuquerque et al., 2010). This suggests that the PMC was operated under appropriate conditions to select for PHA accumulating organisms.

In addition, ammonia consumption was mostly observed during the famine phase. This suggests an inability of the culture to grow in the beginning of the cycle, as observed for aerobic MMCs by Albuquerque et al. (2010), and is due to the physiological adaptation of cells after a starvation period. This finding likely contributed towards the selection of PHA accumulating organisms, since only the organisms with stored carbon would have been able to grow during the famine phase.

During the enrichment period, it was observed that the culture also accumulated carbohydrates. Initially, the carbohydrates were present, but did not vary in abundance (Figure 2.1a). However, along the enrichment phase, the PMC consumed the carbohydrates during the feast phase and restored them in the famine phase (Figure 2.1c). It should be noted that the method utilized to

quantify total carbohydrates accounts not only for bacterial glycogen, but also for algae starch (Ike et al., 1999). For this reason, changes in the PMC carbohydrate pool could be due to the activity of both bacteria and algae. However, the carbohydrate profile is linked to the PHA profile in a similar manner as glycogen accumulating organisms (GAOs) in anaerobic/aerobic FF cycles (Oehmen et al., 2005; Bengtsson, 2009). This suggests that bacterial glycogen was used as a pool of energy that potentiated acetate uptake in the feast phase, leading to PHA production. In the famine phase, glycogen appeared to be restored via PHA oxidation.

It is likely that this PHB consumption and concomitant glycogen storage during the famine phase was possible due to oxygen produced by algae. PHB consumption leads to the production of NADH that must be reoxidized for the cell metabolic flux to be constant. Since electron acceptors were not provided, we hypothesize that oxygen produced by algae led to the oxidation of reducing equivalents by bacteria. The fact that the measured oxygen level in the reactor was zero does not necessarily indicate that there was no oxygen available in the reactor, but that all of the oxygen produced by algae was readily consumed by bacteria. For this reason, the impact of aeration on the PMC was tested in order to establish the culture's capacity to metabolize oxygen.

2.3.2 Oxygen contribution to the PMC feast and famine profile

Batch tests were performed in dark conditions with aeration (Figure 2.2a) to estimate the culture's aerobic activity, and compared to control tests in light-argon conditions (Figure 2.2b).

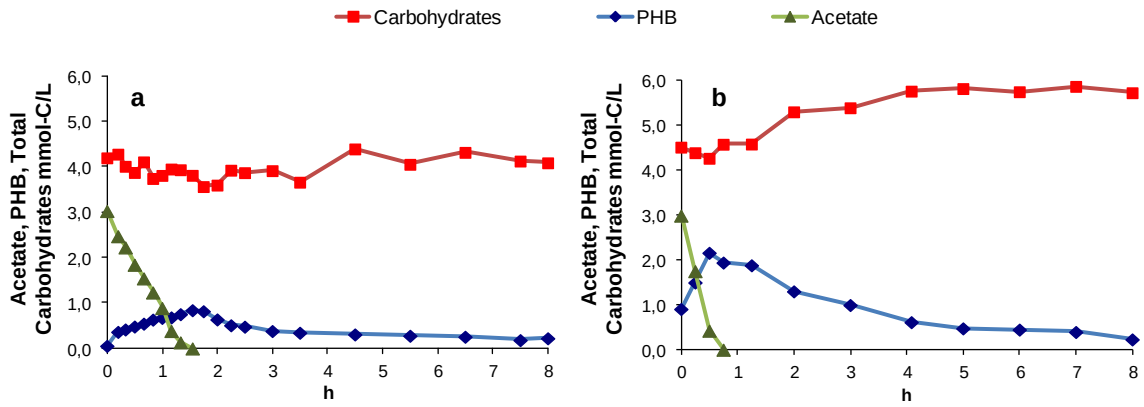

Figure 2.2 – Batch tests in: a) Dark-Aerated conditions, b) Light-Argon conditions.

Under ~90% oxygen saturation and the absence of light, the kinetic parameters of the culture were substantially reduced in comparison to operation under light-argon conditions (Table 2.1).

Table 2.1 - Kinetic and stoichiometric parameters of batch tests performed with the PMC in different experimental conditions. Tests: A) Light-Argon conditions in both the feast and famine phases; B) Dark-Aerated conditions in both feast and famine phases; C) Dark-Argon condition in the feast phase and Light-Argon in the famine phase. Values in brackets are the standard deviation and n the number of experiments used for the calculations.

	A		B		C	
	Light-Argon		Dark-Aerated		Dark-Argon	Light-Argon
	Feast	Famine	Feast	Famine	Feast	Famine
	n = 4	n = 3	n = 2	n = 2	n = 2	n = 2
q_P	1.2 (0.19)	-0.35 (0.15)	0.19 (0.00)	-0.25 (0.05)	0.35 (0.14)	-0.34 (0.10)
q_{Carbs}	-0.33 (0.16)	0.24 (0.05)	-0.10 (0.04)	0.13 (-)	-0.48 (0.13)	0.23 (0.02)
$-q_S$	2.0 (0.23)	-	1.0 (0.05)	-	0.33 (0.09)	-
$Y_{PHB/S}$	0.61 (0.11)	-	0.18 (0.01)	-	1.04 (0.13)	-
$Y_{Carbs/S}$	-0.17 (0.09)	-	-0.09 (0.03)	-	-1.47 (0.24)	-
$Y_{Carbs/PHB}$	-	0.75 (0.34)	-	0.43 (0.10)	-	0.70 (0.26)
$Y_{PHB/Carbs}$	-	-	-	-	0.72 (0.12)	-

q_P in Cmol PHB/Cmol X d; q_{Carbs} in Cmol Carbs/Cmol X d; $-q_S$ in Cmol Acet/Cmol X d; $Y_{PHB/S}$ in Cmol PHB/Cmol Acet; $Y_{Carbs/S}$ in Cmol Carbs/Cmol Acet; $Y_{Carbs/PHB}$ in Cmol Carbs/Cmol PHB; $Y_{PHB/Carbs}$ in Cmol PHB/Cmol Carbs

The acetate uptake rate of the culture dropped by 50%, suggesting that the ATP produced via the PMC's photo-metabolism is essential for accelerating acetate uptake. Since the PMC was acclimatized to minimal levels of oxygen and had an alternative means of obtaining energy (photosynthetically), its systems of energy production by oxidative phosphorylation were most likely sub-expressed. Therefore, in the dark-aerated tests, the sole source of energy for the PMC came from those sub-expressed systems, which may have resulted in low energetic availability for acetate uptake. Nevertheless, the possibility that part of the acetate consumption and PHB production in the PMC was due to heterotrophic bacteria (the bacteria commonly found in MMC) could not be excluded.

In the dark-aerated test, an inferior amount of PHB was accumulated and less carbohydrate was utilized, as reflected in the lower values of $Y_{PHB/S}$ and $Y_{Carbs/S}$ that decreased to one-third and one-half, respectively, as compared to the Light-Argon test (cf. Table 2.1). This likely resulted from the higher oxygen availability, which may have caused the respiration of most of the acetate, lowering the amount of carbon accumulated as PHB and the need for ATP production via bacterial glycogen. These results suggest that the illuminated environment of the PMC is largely responsible for substrate uptake, glycogen utilization and accumulation of carbon as PHB, and not the oxygen produced by algae.

2.3.3 PMC carbohydrate metabolism

The importance of carbohydrates in the PMC metabolism was further analysed in two batch tests with periods without light. In the first test, the feast phase began in light-argon conditions and the light was switched off after acetate was depleted (Figure 2.3a).

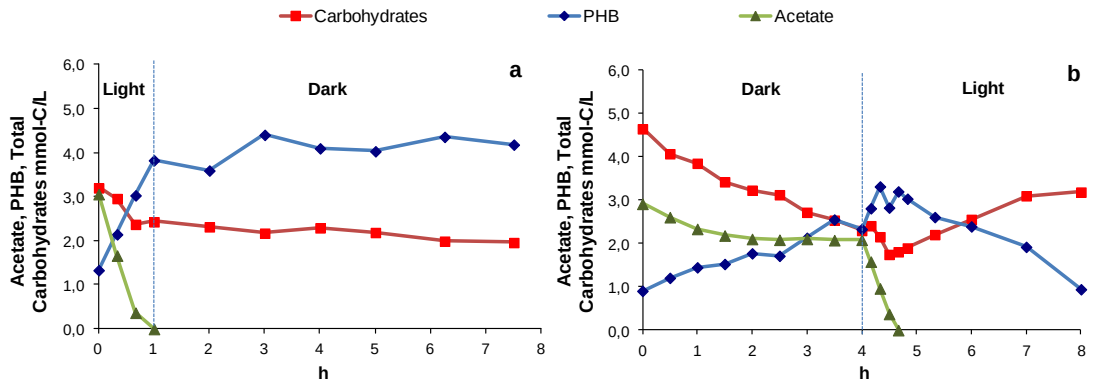

Figure 2.3 – Batch tests in: a) Light-Argon conditions in the feast followed by Dark-Argon in the famine; b) Dark-Argon conditions in the feast, followed by Light-Argon conditions during the consumption of the remaining acetate and during the famine. Dashed lines indicate the transition in illumination conditions.

When the PMC was subjected to dark-argon conditions in the famine phase, there was an inversion of the culture's behaviour as compared to an illuminated famine phase (Figure 2.2b). During the dark famine phase, carbohydrates were consumed and PHB was accumulated at much lower rates (less than 5%) as compared to those in the feast phase. This behaviour may be explained by the fact that oxygen could not be produced by the algae in the dark, and thus there was no electron acceptor available for the bacteria to consume PHB and restore glycogen. Instead, the PMC likely consumed glycogen to generate energy for cell maintenance purposes. Without available electron acceptors, PHB was produced from glycogen consumption as a means of maintaining the cellular carbon and energy homeostasis. The calculated $Y_{PHB/Carbs}$ for the dark famine was 0.94 ± 0.11 Cmol PHB/Cmol Carbs (result from 2 batch tests), which corroborates the hypothesis that glycogen was mainly converted into PHB. Glycogen conversion to PHA during anaerobic cell maintenance processes has previously been observed by glycogen accumulating organisms (Filipe et al., 2001).

In the second batch test performed to analyse the PMC carbohydrate metabolism, dark-argon conditions were provided for the first 4 hours, which was followed by illumination for an additional 4 hours (Figure 2.3b). The carbohydrate consumption rate increased 45% during the dark feast phase, as compared to illuminated feast conditions (Table 2.1). Acetate was slowly and partially taken up in the beginning of the test, and its consumption ceased shortly thereafter. The higher amount of energy initially obtained via carbohydrate consumption likely explains

the initial acetate uptake. However, the carbohydrate consumption rate decreased with its storage level, likely leading to insufficient energy for acetate uptake. Carbohydrates were still consumed for cell maintenance, since they represent the only energy source available without illumination. It was also confirmed that phosphorus release was not observed during light-argon and dark-argon conditions, which would have been expected from a polyphosphate accumulating organism (PAO) type of metabolism (data not shown).

PHB production was also observed throughout the entire dark feast phase, with its accumulation being initially associated to the bacterial glycogen consumption and acetate uptake. After acetate uptake ceased and only glycogen was consumed, PHB accumulation was still observed. Despite the fact that the acetate uptake rate and the PHB production rate were low (cf. Table 2.1), the PMC could still perform those processes in dark anaerobic conditions as a result of its ability to utilize the stored carbohydrates.

The $Y_{Carbs/S}$ of the PMC from the beginning of the dark feast phase until the acetate uptake ceased was 1.47 ± 0.24 Cmol Carbs/Cmol Acet, a value much higher than the 0.17 ± 0.09 Cmol Carbs/Cmol Acet presented in illuminated feast phases, and comparable with $Y_{Gly/S}$ values of acetate-fed glycogen accumulating organism (GAO) cultures operated in FF anaerobic/aerobic cycles (0.92-1.68 Cmol Carbs/Cmol Acet, see Oehmen et al., 2010).

The relatively high yield of carbohydrate consumption per acetate consumed of the PMC in relation to the $Y_{Gly/S}$ of GAO cultures may also be due to the contribution of the algae starch. In dark anaerobic conditions, algae can ferment accumulated starch, obtaining energy as ATP and reducing equivalents (Gfeller and Gibbs, 1984; Ohta et al., 1987). Starch degradation can result in the formation of different fermentation products, such as: H_2, CO_2, acetate, ethanol, formate, glycerol, lactate and butanediol (Klein and Betz, 1978; Gfeller and Gibbs, 1984; Ohta et al., 1987). During the dark anaerobic feast phase, residual levels of lactate and glycerol were detected, with a higher predominance and accumulation of glycerol (data not shown). In spite of the fact that formate, acetate and ethanol are usually the most representative starch fermentation products (Gfeller and Gibbs, 1984), their production was not observed. However, acetate production is difficult to distinguish from the acetate already added as substrate. If its production was occurring, the yield of carbohydrates per acetate taken up would increase relative to GAO metabolic model predictions. Concerning the other two products, as their production is dependent on the algae species and on the operational conditions, they may not have been detected because they are not the starch fermentation products of the algae present in this consortium.

Accordingly, the yield of PHB produced per carbohydrates consumed (0.72 ± 0.12 Cmol PHB/Cmol Carbs) is inferior to the yield of PHB per glycogen observed by most GAO cultures

(1.10-1.32 CmolPHB/Cmol Gly, see Oehmen et al., 2005; Bengtsson, 2009), another possible indication of algae activity. As PHB is only produced by the bacteria, taking into account the algae starch consumption would lead to a lower yield of PHB per carbohydrate.

However, it should be noted that in previous studies with GAO cultures, anaerobic glycogen consumption also led to PHV production in addition to PHB. According to the stoichiometry of the glycolysis process by GAOs, the PHB:PHV ratio should be 3:1 (Zeng et al., 2002). In our test, the production of PHV was not observed in the dark feast phase, only PHB was produced. This could be due to the low PHB production observed in this test (Δ 1.2 mmolC PHB/L), and since the PHV would theoretically be 3 times lower, it would not be detectable by the analytical method used (Detection limit 1 mmolC PHV/L).

After illumination of the system, the PMC resumed its typical profile under light-argon conditions: acetate was rapidly taken up, carbohydrates were consumed and PHB was accumulated (Figure 2.3b). During the illuminated famine phase, PHB was consumed and carbohydrates restored. The behavior of the culture in this dark/light test seems very promising for the development of a PHA producing system. The PMC can still take up acetate and produce PHB using carbohydrates during the dark feast phase, which is accelerated once light is provided, suggesting that the PMC could be subjected to dark/light cycles and maintain its viability and PHA accumulation capacity. This would be of great interest if the illumination conditions provided were similar to the daily solar pattern.

2.3.4 PMC microbial characterization

The PMC studied in this work consisted of a consortium of bacteria and algae, which were mostly aggregated in flocs or small granules, although some biomass was suspended as pin-point flocs. These aggregates were constituted by a nucleous of algae surrounded by a much greater number of bacteria. This aggregation of bacteria to algae can be naturally explained by the fact that algae are the producers of the oxygen that bacteria consume. Also, bacterial internal PHA granules were identified by Nile Blue staining, where many different bacterial morphological groups appeared to accumulate this polymer.

FISH analyses were performed to identify the bacterial fractions of the PMC. The general proteobacterial FISH probes showed the presence of both *Alphaproteobacteria* and *Gammaproteobacteria*, with the latter being slightly higher in abundance. Two main morphotypes were detected by microscopic observations of the samples, where most rods were identified as *Alphaproteobacteria* and cocci as *Gammaproteobacteria*, with both groups being able to accumulate PHA, as indicated by Nile Blue staining.

Within the PNS bacterial group, the presence of some *Rhodobacter* and a few *Rhodopseudomonas* were detected, which indicates a small contribution of this group to the culture's bacterial diversity. A fraction of the bacterial culture may have been purple sulfur bacteria, given that these organisms are *Gammaproteobacteria*, and as previously indicated, *Gammaproteobacteria* are dominant in the culture. However, since FISH probes targeting the purple sulfur bacteria are not currently available, the specific identification of these organisms could not be established. Future work will involve designing new probes and characterizing further the culture's bacterial fraction.

All of the other probes that were tested did not show a positive signal, including known GAOs, PAOs and PHA producers found in aerobic MMCs. This is particularly significant considering that the PMC bacterial faction seems to behave similarly to a GAO culture in dark anaerobic conditions. Whether this GAO behavior is effectively due to heterotrophic GAOs or to a new, yet undescribed GAO active under photosynthetic conditions, could not be determined by FISH. Nevertheless, it is known that e.g. Purple sulfur and non-sulfur bacteria can store carbohydrates and PHA as carbon reserves (Imhoff, 2006). It can be hypothesized that photosynthetic bacteria make use of carbohydrates as an energy source for taking up external carbon and restore their carbohydrate pool from accumulated PHB, thus displaying a GAO phenotype. This would suggest that phototrophic GAOs are capable of utilizing a myriad of metabolic pathways, such as photosynthesis, or its internal reserves (or a combination of both) according to the environment conditions, e.g. aerobic, oxygen limited, dark or illuminated. However, it is worth noting that if the activity is instead due to ordinary heterotrophic GAOs, this would still represent a new, previously unidentified GAO that can survive and perform glycogen cycling using limited levels of oxygen in the famine phase, suggesting a very high affinity coefficient for oxygen.

2.3.5 PMC PHB accumulation capacity

To assess the PHB accumulation capacity of the PMC in illuminated non-aerated conditions, two batch tests were performed. In one of the batch tests, a single pulse of a concentrated acetate solution was added, making the initial substrate concentration five times higher than the typical concentration in the PMC (Figure 2.4a). This test also evaluated the effect of higher amounts of acetate (15 C-mM) on the culture.

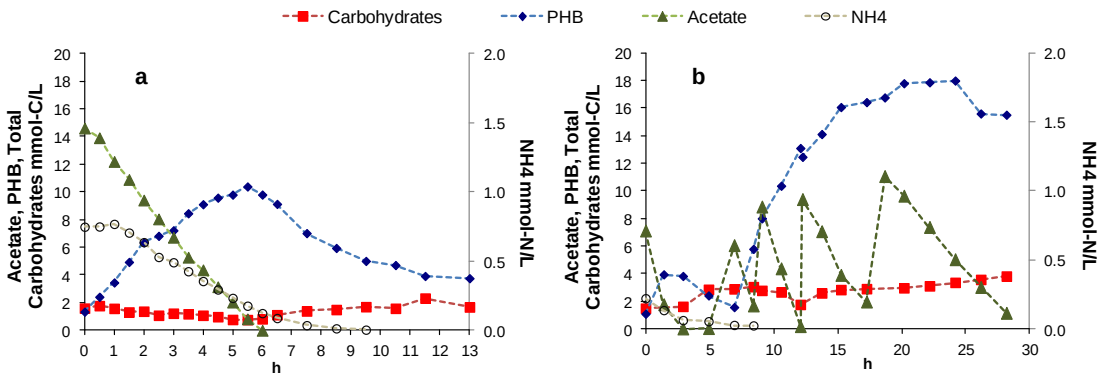

Figure 2.4 – PHB accumulation batch tests in Light-Argon conditions: a) addition of a concentrated single pulse of acetate; b) addition of multiple pulses of acetate

Linear acetate consumption was observed at half the typical rate (1.0 ± 0.02 Cmol Acet/Cmol *X* d). This lower acetate uptake rate could have been caused by the higher substrate concentration, leading to inhibition. However, as the acetate concentration decreased, an increase in the uptake rate was not observed, as would be expected when the substrate concentration reaches non-inhibitory levels. Instead, the acetate uptake rate remained constant until it was fully consumed. It is more likely that the lower acetate uptake rate was due to the relatively low initial carbohydrate storage, which resulted in an inferior carbohydrate consumption per substrate added, and thus, in a lower energetic availability for acetate uptake. The amount of carbohydrates utilized for acetate uptake was only 0.07 ± 0.01 Cmol Carbs/Cmol Acet in this test, a value that is less than half of the typical level (0.17 ± 0.09 Cmol Carbs/Cmol Acet).

The PHB accumulation rate was constant during the first 2h of the batch test with 15 C-mM of acetate, decreasing afterwards by 50%. This suggests that a portion of the PMC population had become saturated in PHB, lowering the number of organisms accumulating PHB. However, biomass growth commenced simultaneous to the decrease in PHB accumulation rate. In tests with 3 C-mM acetate, growth began only after all substrate had been depleted (i.e. tests had shorter feast phases). Since part of the acetate taken up was being diverted for growth during the extended feast phase, this likely contributed to the 50% decrease in the PHB accumulation rate.

After all of the acetate was consumed, the PHB accumulated was 14% ± 0.5%, which represents a considerable increase as compared to the typical PHB levels achieved with a feed of 3 C-mM of acetate (3 ± 1%).

During this test, the PHB consumption after the depletion of acetate was also investigated. The maximum specific PHB consumption rate was 0.71 ± 0.06 Cmol PHB/Cmol *X* d, two times faster than the average rate presented in the 3 C-mM tests (cf. Table 2.1). The fact that the PHB consumption rate increased with the increase of the cell PHB content is an indication that its

consumption kinetics is ruled by the internal PHB concentration (following the equation $q_p(t) = k_p.[PHA]^{2/3}$ described by Murnleitner et al. (1997)). Despite the fact that the measured oxygen levels in this test were also zero, the oxygen generated by the algae met the PMC oxygen requirements during the famine phase.

In order to test the maximum PHA accumulation level achievable by the culture, a second test was performed with the addition of multiple pulses of acetate (Figure 2.4b). Pulse-feeding was performed in order to prevent against possible substrate inhibition and to ensure that the acetate was not limiting the PHA accumulation. A typical cycle of PHB accumulation/consumption was first performed, with the purpose of consuming all of the remaining ammonia and thus, preventing the diversion of acetate for cell growth. After this cycle, four more acetate pulses were added. The acetate uptake rate decreased as the culture became more saturated with PHB, eventually achieving a plateau at a maximum PHB content of 20% ± 0.2%. It should be noted that this value takes into account both bacteria and algae biomass, suggesting that the culture would contain higher amounts of PHB per bacterial fraction. During the PHB accumulation phase (considering the 2nd to 4th pulse), the culture presented a $Y_{PHB/S}$ (0.70 ± 0.04 Cmol PHB/Cmol Acet) slightly higher than the yields presented by the PMC in the single pulse accumulation test and in the 3 C-mM tests.

Comparing this system with other MMC systems enriched with acetate as the sole carbon source, Serafim et al. (2004) observed similar PHB yields per acetate using aerobic conditions, with values of 0.70 and 0.78 Cmol PHB/Cmol Acet for tests with single and multiple pulses of acetate, respectively. However, in GAO cultures with alternating anaerobic/aerobic conditions, the PHB yield per acetate ranged from 1.2 Cmol PHB/Cmol Acet in anaerobic single acetate pulse tests (1.5 Cmol PHA/Cmol Acet when considering both PHB and PHV formation) to 0.5 Cmol PHB/Cmol Acet in aerobic tests with multiple acetate pulses (Bengtsson, 2009). The fact that our PHB yield per acetate is in a similar range as previous studies shows that achieving PHA production without aeration inputs is indeed feasible and could present a potentially interesting process option.

The maximum specific PHB production rate obtained with the mixed photosynthetic culture was 1.1 ± 0.1 Cmol PHB/Cmol *X* d in both the accumulation tests and in the 3 C-mM tests. These rates are 10 times inferior to those obtained by Serafim et al. (2004) under aerobic conditions (9.6 – 12 Cmol PHB/Cmol *X* d) and 10 to 20 times inferior than in Bengtsson (2009) with GAOs (10-20 Cmol PHB/Cmol *X* d, depending on the initial glycogen pool). The lower rate is likely due to the fact that energy production in the PMC is dependent on photosynthetic systems, and the ATP availability is directly linked to the light intensity provided. Higher light intensities could accelerate the PMC metabolism and increase the PHB production rate to higher

levels. However, the photosynthetic PHB production rates may not need to equal aerobic rates in order to ensure that the photosynthetic process is interesting from an industrial point of view. Future work should compare the costs of aeration with those associated with utilizing solar light in order to evaluate the economic feasibility of photosynthetic PHB production. Since the PMC can produce a similar quantity of PHA per carbon source in a non-aerated system as compared to an aerated system, photosynthetic PHA production is worthy of further investigation for process optimization.

2.4 CONCLUSIONS

In this work, a mixed microbial culture PHA production system that does not require aeration is proposed. The photosynthetic consortium of bacteria and algae accumulated PHA under feast/famine conditions, and has thus far reached a maximum PHB content of 20%. The yield of PHA production per substrate consumed was in the same range as aerobic PHA producing MMC. The utilization of mixed photosynthetic cultures surges as a new and alternative system for mixed culture PHA production, with the potential advantage of minimized costs through utilizing solar energy.

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3

Effect of dark/light periods on the polyhydroxyalkanoate production of a photosynthetic mixed culture

Summary *This work studied the possibility of operating a viable polyhydroxyalkanoate (PHA) producing photosynthetic mixed culture (PMC) under dark/light periods without aeration. The culture was subjected to a feast and famine regime, being fed in the dark phase and entering into famine during the light phase. Throughout consecutive feast and famine dark/light periods, the PMC became enriched in PHA accumulating organisms, where non-PHA producing algae that can grow under continuous illumination were out-competed. The very low algae levels enabled greater light and carbon source availability for PHA accumulating bacteria, leading to higher metabolic rates and PHA levels. The PMC reached a PHA content of 30% PHA/VSS, and doubled its specific PHA production rate in relation to PMCs operated previously under continuous illumination. This new process takes a further step towards operating a more cost effective PMC system for PHA production, opening up the possibility for direct sunlight utilization in the future.*

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3.1 INTRODUCTION

In the last decades, we have witnessed an increasing demand for environmentally friendly materials that could replace the non-degradable plastics chemically synthesized from finite oil reserves. To address this demand, researchers have focused their attention on polyhydroxyalkanoates (PHAs), which are natural biodegradable polyesters internally synthesized by some microorganisms as carbon reserves. These biopolymers present thermoplastic properties similar to conventional plastics, making them potential replacement materials for petrochemically based plastics (Braunegg et al., 1998; Verlinden et al., 2007).

To date, PHA is already industrially produced using pure cultures and refined substrates under sterile operating conditions. However, in order to compete with the low price of traditional plastics, the current high costs of industrial PHA production must decrease (Reis et al., 2011). To make this biopolymer a more economically accessible material, several studies using mixed microbial cultures (MMC) fed with low/zero cost substrates have been proposed. Different feedstocks like sugar cane molasses (Albuquerque et al., 2010; Bengtsson et al., 2010), olive oil mill pomace (Waller et al., 2012), mixtures of sludge and food waste (Chen et al., 2013), and wastewater from paper mill effluents (Bengtsson et al., 2008; Jiang et al., 2012) have been used as substrates for PHA production and demonstrated comparable yields to PHA production with pure cultures, thus contributing to a decrease in PHA production costs while simultaneously treating polluted streams.

The selection of a MMC with a high PHA storing capacity using such agro-industrial wastes or by-products is usually performed through transient feeding conditions of the desired feedstock, in a strategy designated as Feast and Famine (FF). By feeding the culture intermittently, organisms that accumulate the substrate as PHA during the feed phase will be favoured during the famine phase, since only the organisms containing storage products will be able to grow in the absence of external substrate, thus enriching the MMC in PHA accumulating organisms (Reis et al., 2003). Results from the application of this strategy have been promising so far, high polymer content has been achieved, reaching maximum values of 77% PHA content when feeding paper mill wastewater (Jiang et al., 2012). Moreover, diverse monomeric compositions have been obtained, with co-polymers of short and medium chain length monomers being simultaneously produced, conferring broader thermal and mechanical properties to PHA (Pisco et al., 2009; Albuquerque et al., 2011).

Despite the efforts to develop less expensive PHA production systems, all of these studies utilizing mixed microbial cultures require intensive aeration, which substantially increases operational costs (Rosso et al., 2008). To avoid these aeration costs, a new MMC PHA

production system has been recently proposed using photosynthetic microorganisms (Fradinho et al., 2013). In this photosynthetic system, the capability of microorganisms to accumulate PHA was explored, with the advantage that these photosynthetic organisms can obtain energy from light, thus, not requiring aeration for ATP production. Using a FF strategy and with constant illumination, Fradinho et al. (2013) enriched a photosynthetic mixed culture (PMC) composed of a consortium of bacteria and algae, with bacteria accumulating PHA in the feast phase, and consuming it in the famine phase using the oxygen produced by the algae. The PMC accumulated up to 20% PHA/VSS with acetate as the carbon source, with a yield of PHA per substrate of 0.70 Cmol PHA/Cmol Acet, which is similar to the yields obtained with aerobic MMC selected under feast/famine conditions.

Despite the elimination of aeration costs, the constant illumination of the proposed photosynthetic system would also add associated costs. One way of minimising the operational costs of the PMC in future applications would be the use of solar light as the illumination source. For this reason, it is critical to assess the impact of alternating dark/light periods on the performance of the PMC, thus evaluating if the photosynthetic culture can endure dark periods as would occur with direct sunlight utilization. In a batch test, Fradinho et al. (2013) tested the PMC behaviour in response to dark anaerobic conditions, and observed that internally stored carbohydrates were utilised as an energy source for acetate uptake, resulting in PHA production. However, long-term studies with sequential dark/light cycles are needed in order to determine if this capacity can be exploited to operate a PMC with effective PHA producing characteristics with reduced illumination periods. Therefore, in this work, we evaluate the possibility of enriching a viable photosynthetic mixed PHA accumulating culture in a regime with dark/light periods, and assess the impact of the illumination pattern on the culture community and its PHA accumulating capacity. The effect of this operational strategy was evaluated through assessing the stoichiometric and kinetic parameters of the PMC, which was accompanied by microscopic examination of the culture.

3.2 MATERIALS AND METHODS

3.2.1 Mixed photosynthetic culture operation under dark/light cycles

The inoculum for the PMC studied in this work was obtained from a photosynthetic consortium of bacteria and algae that was operated with continuous illumination (1.3 W/L of culture broth) in a feast and famine regime, using acetate as carbon source (6 C-mM in the beginning of the feast phase) and containing a biomass concentration of 2.7 g VSS/L. The PMC operation under dark/light periods was performed in a 500 mL sequencing batch reactor (SBR) with 8h cycles, where in the first 4h of the cycle the reactor was under dark conditions, and in the last 4h hours,

the reactor was externally illuminated by a tungsten lamp (60 W) at a light intensity of 48 W/m^2, which corresponded to a volumetric intensity of 1.3 W/L of culture broth. This volumetric light intensity was the same used in a previous work with continuous illumination (Fradinho et al. 2013) and that demonstrated to be adequate to enrich a PHA accumulating photosynthetic mixed culture. Acetate was used as carbon source, at a concentration of 3 C-mM in the beginning of the dark phase. A lower initial acetate concentration was fed in this system in order to ensure an adequate F/F ratio, since acetate uptake kinetics were slower under dark conditions as compared to illuminated conditions in short-term batch tests (Fradinho et al., 2013). Maintaining a low F/F ratio has previously been shown to be of high importance towards enriching PHA accumulating organisms (Albuquerque et al. 2010).

At the beginning of the dark phase of each cycle, the SBR was fed with equal amounts of culture medium (14 mL containing per liter: 0.8 g $MgSO_4.7H_2O$, 1.6 g NaCl, 1.1 g NH_4Cl, 0.2 g $CaCl_2.2H_2O$, 8.2 g NaAcetate.$3H_2O$, 20 mL iron citrate solution (1.0g/L), 4 mL trace element solution) and phosphate medium (14 mL containing per liter: 0.13 g KH_2PO_4 and 0.17 g K_2HPO_4), which corresponds to an organic loading rate (OLR) of 0.65 g COD/L d. This value is in line with the OLR applied in Fradinho et al. (2013). Despite being lower than the OLRs usually applied in aerobic systems (Dionisi et al., 2006; Albuquerque et al., 2010; Villano et al., 2010), it is appropriate for the slower kinetics of photosynthetic microorganisms as demonstrated in Fradinho et al. (2013), enabling the establishment of a FF regime and the selection of a PHA accumulating culture.

At the end of the illuminated phase of each cycle, 28 ml of the continuously stirred PMC were wasted, resulting in a hydraulic retention time (HRT) and sludge retention time (SRT) of 6 days. Temperature was controlled at 30ºC and argon was continuously sparged (10mL/min) to prevent surface aeration. pH was controlled at 6.5 using 0.5 M HCl.

3.2.2 PHA accumulation test

After 3 SRT of operation under the dark/light period regime, and with more than 1 SRT of stable biomass concentration, a batch test was performed to evaluate the culture's maximum PHA accumulation capacity. The test started as a routine cycle (feeding 14 ml of culture medium and 14 ml of phosphate medium), but instead of beginning in dark conditions, the test was continuously conducted under the illumination conditions described in section 3.2.1. Pulses of an acetate solution (3.75 C-mol/L) were added throughout the test, preventing carbon depletion.

3.2.3 Analytical Methods

PHA determination was performed by gas chromatography using the method described in Fradinho et al. (2013). The acetate concentration was determined by high-performance liquid chromatography (HPLC) using an IR detector and a BioRad Aminex HPX-87H column. 0.01 N sulfuric acid was used as eluent with an elution rate of 0.6 mL/min and a 50ºC operating temperature.

Total carbohydrates hydrolysable to glucose were determined using the method described by Lanham et al. (2012), with minor modifications. In brief, culture samples were centrifuged, and the pellet was washed twice with 0.9 % NaCl and lyophilized overnight. The biomass was then mixed with 2 mL of 0.6 M HCl and subjected to digestion for 2h at 100ºC. The supernatant was filtered (0.2 µm membrane) and glucose was analyzed by HPLC as described above, but using an elution rate of 0.5 mL/min and a 30ºC operating temperature.

Volatile suspended solids (VSS) were determined according to Standard Methods (APHA, 1995). The light intensity provided during the tests was measured using a Li-COR light meter LI-250 A equipped with a pyranometer sensor LI-200 SA.

3.2.4 Microbial characterization of the PMC

Biomass samples were collected throughout the culture enrichment and were examined under the microscope for morphological observation, visualization of intracellular PHA granules (through Nile blue staining) and for bacterial community analysis by fluorescence *in situ* hybridization (FISH). Nile blue staining was performed on wet biomass according to Bengtsson et al. (2008). For FISH analysis, sludge samples were fixed with paraformaldehyde as described by Nielsen et al. (2009). Details on oligonucleotide probes are available at probeBase (Loy et al., 2007), unless indicated. The following specific oligonucleotide probes were employed: ALF969 for *Alphaproteobacteria* (Oehmen et al., 2006), BET42a for *Betaproteobacteria*, GAM42a for *Gammaproteobacteria*, Delta495a for *Deltaproteobacteria*, Rhodopseud for *Rhodopseudomonas* and GAOmix (GB G2 + GAOQ989) for *Competibacter phosphatis*. Each specific probe was applied with a Cy-3 label, together with a FITC-labelled EUBMIX probe for all *Bacteria* (EUB338 and EUB338-II and III). An Olympus BX51 epifluorescence microscope was used for the microscopic observations of biomass samples.

3.2.5 Calculation of kinetic and stoichiometric parameters

The biomass PHA content was calculated as a percentage of VSS on a mass basis ($\%PHA = 100 \times g\ PHA/\ g\ VSS$), where VSS includes active biomass (X), PHA and total carbohydrates. Active biomass was calculated by subtracting PHA and total carbohydrates from VSS.

The maximum specific substrate uptake rate (-q_S in Cmol Acet/Cmol X d), maximum specific PHA production rate (q_P in Cmol PHA/Cmol X d) and maximum specific carbohydrate utilization rate (q_{Carbs} in Cmol Carbs/Cmol X d) were determined by adjusting a linear regression line to the experimental concentrations determined over time and dividing the slope of the fitting at time zero by the concentration of active biomass at that point.

The yields of PHA per substrate consumed ($Y_{PHA/S}$ in Cmol PHA/Cmol Acet) was calculated by dividing the amount of PHA formed by the amount of acetate consumed. The yield of PHA per total carbon consumed ($Y_{PHA/C}$ in Cmol PHA/Cmol Total Carbon) was calculated by dividing the amount of PHA formed by the amount of acetate and carbohydrates consumed.

The specific light intensity of the culture (W/g X), was calculated by dividing the volumetric intensity of the culture broth (1.3 W/L) by the active biomass concentration (g X/L).

3.3 RESULTS AND DISCUSSION

3.3.1 Photosynthetic mixed culture operation under dark/light cycles

In this study, a PMC consisting of bacteria and algae that had been enriched under constant illumination was subjected to alternating dark/light conditions in a FF regime, with the culture being fed at the beginning of the dark phase. Figure 3.1 shows the evolution of the culture's performance during an adaptation period of 16 days to the transient illumination conditions. It can be observed that on the 1st day, the PMC was capable of utilizing carbohydrates during the dark phase, likely using it as energy source for acetate uptake (Figure 3.1a). It should be noted that in dark anaerobic conditions, the PMC cannot produce energy photosynthetically, therefore, the stored carbohydrates serve as the only energy source. Additionally, some PHA production was also observed, in the form of a homopolymer of hydroxybutyrate units (PHB), which can be associated with the acetate taken up and the carbohydrate consumed. Most of the acetate was consumed during the dark phase, such that at the beginning of the light phase, the culture quickly entered into famine and started to consume its PHB reserves.

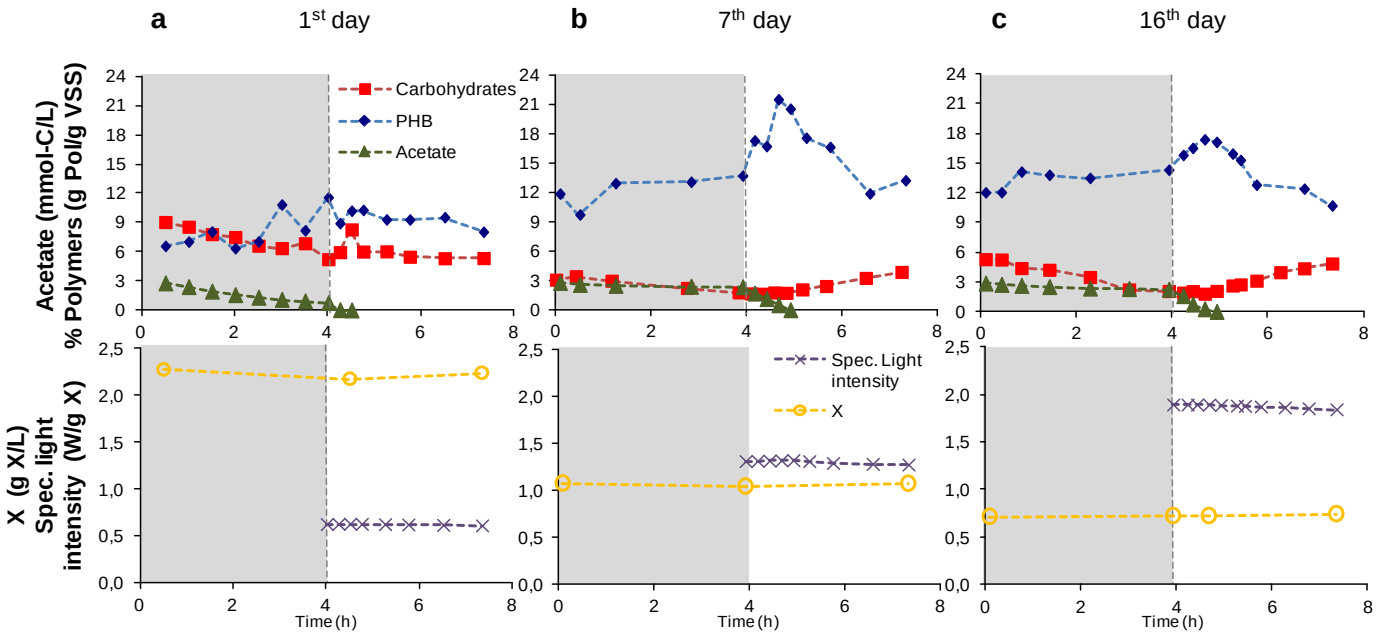

Figure 3.1 – Evolution of the cultures' behavior during the adaptation period to dark/light periods in an 8h cycle SBR when subjected to feeding in the dark phase (grey area) and famine during the light phase (white area).

Throughout the following adaptation period (Figure 3.1b-c), the culture continued to consume carbohydrates as an energy source for acetate uptake with some PHB production occurring during the dark phase (Table 3.1). Despite the fact that similar acetate consumption rates were maintained through this period (~0.23 ± 0.01 Cmol Acet/Cmol *X* d), lower amounts of acetate were being consumed in the dark phase, mostly due to the lower biomass concentrations in the PMC at the end of the adaptation period (Figure 3.1a-c). This led to higher concentrations of acetate at the beginning of the illuminated phase. As a result, by the end of the adaptation period, 76 % of the total acetate consumption was occurring in the illuminated phase, while in the first day, it was only 29%.

After entering the light phase, the activity of the PMC increased considerably, with a tenfold increase in the acetate consumption and PHB production rates in the illuminated feast phase at the end of the adaptation period in comparison to the dark period (Table 3.1). This accelerated activity resulted from the ATP that became available once the PMC photosystems became active under illumination. For this reason, during the illuminated feast phase, no carbohydrate consumption was observed, since the PMC no longer required it for energy generation.

Table 3.1 – Kinetic and stoichiometric parameters of the PMC performance during the adaptation period and in the PHA accumulation batch test. Fradinho et al. (2013) PHA accumulation tests results are also indicated for comparison purposes. Values in brackets are the error determined with base on the error of the fitting when calculating maximum specific rates. * Values in brackets are the standard deviation calculated from 4 experiments

		This Work				Fradinho et al. (2013)		
		Adaptation period				a*	b	c
		1st day	7th day	16th day				
Dark phase	q_P	0.32 (0.13)	0.14 (0.04)	0.15 (0.09)	Accumulation test	3 mM-C Test	Accumulation test multi pulse	Accumulation test single pulse
	$-q_{Carbs}$	0.27 (0.02)	0.11 (0.00)	0.21 (0.02)				
	$-q_S$	0.22 (0.01)	0.27 (0.00)	0.21 (0.02)				
Light feast phase	q_P	-	2.80 (0.27)	1.60 (0.10)	2.23 (0.15)	1.2 (0.19)	1.08 (0.10)	1.04 (0.05)
	$-q_{Carbs}$	-	0.04 (0.00)	0.06 (0.00)	0.11 (0.01)	0.33 (0.16)	0.02 (0.00)	0.07 (0.01)
	$-q_S$	0.78 (0.00)	1.35 (0.00)	2.24 (0.17)	2.19 (0.07)	2.00 (0.23)	1.06 (0.00)	1.03 (0.02)
	$Y_{PHB/S}$	-	-	0.71 (0.10)	0.94 (0.09)	0.61 (0.11)	0.70 (0.04)	0.64 (0.04)
	$Y_{PHB/C}$	-	-	0.69 (0.09)	0.90 (0.09)	0.52 (0.11)	0.70 (0.05)	0.60 (0.05)
	Spec. light intensity	0.60	1.27	1.91	1.86	1.05	0.91	0.93

q_P in Cmol PHB/Cmol *X* d; $-q_{Carbs}$ in Cmol Carbs/Cmol *X* d; $-q_S$ in Cmol Acet/Cmol *X* d; $Y_{PHB/S}$ in Cmol PHB/Cmol Acet; $Y_{PHB/C}$ in Cmol PHB/Cmol Total Carbon

The PMC also entered into famine during the light phase, with the previously accumulated PHB being consumed as an internal carbon source and partially converted to carbohydrates. This interconversion of PHB and carbohydrates (carbohydrates to PHB in the feast phase and PHB to carbohydrates in the famine phase), has previously been observed in another mixed photosynthetic culture (Fradinho et al., 2013) and in cultures of glycogen accumulating organisms (GAOs) with anaerobic/aerobic FF cycles (Oehmen et al., 2005; Bengtson, 2009).

Throughout the PMC adaptation period, a decrease in biomass concentration was observed, from an initial 2.3 g *X*/L to 0.7 g *X*/L (Figure 3.1a-c), which stabilized from day 12 on (Figure 3.2).

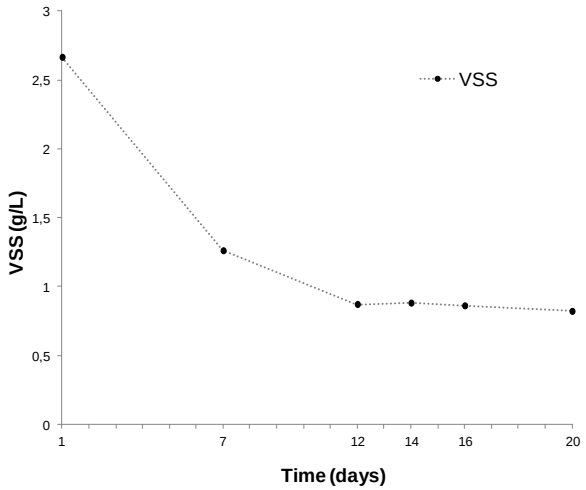

Figure 3.2 – PMC biomass concentration (VSS) evolution during the adaptation period to dark/light periods

Since the adaptation period started with sludge that was acclimatized to 6 C-mM of acetate feeding under constant illumination, a biomass concentration decrease was expected, not only because a lower acetate concentration (3 C-mM) was fed, but also because the illumination period was decreased by half.

Furthermore, Fradinho et al. (2013) obtained a biomass concentration of 1.3 g *X*/L in a PMC fed with the same acetate concentration of this work (3 C-mM), in a system operated under constant illumination. In the present work, the PMC contained half of that biomass concentration (0.7 g *X*/L), which can be explained by the fact that it had only half of the light availability of the Fradinho et al. (2013) culture, and thus, had fewer periods of photosynthetic activity to grow.

Directly associated to the decrease in biomass concentration is the increase of the light availability in the PMC during the light period. The specific light intensity in the culture is shown in Figure 3.1a-c, which tripled during the adaptation period. With the higher light

availability, the culture's photosystems had the means to produce larger amounts of ATP that could be used in the cells' metabolism. In fact, an increase in the specific acetate uptake rate was observed in the illuminated phase throughout the adaptation period, from 0.78 ± 0.00 to 2.24 ± 0.17 Cmol Acet/Cmol *X* d, which can be related to the increased capacity of the culture to produce energy and use it for carbon uptake.

A linear relation was observed between the specific acetate uptake rate in the illuminated phase and the specific light intensity of the culture (Figure 3.3).

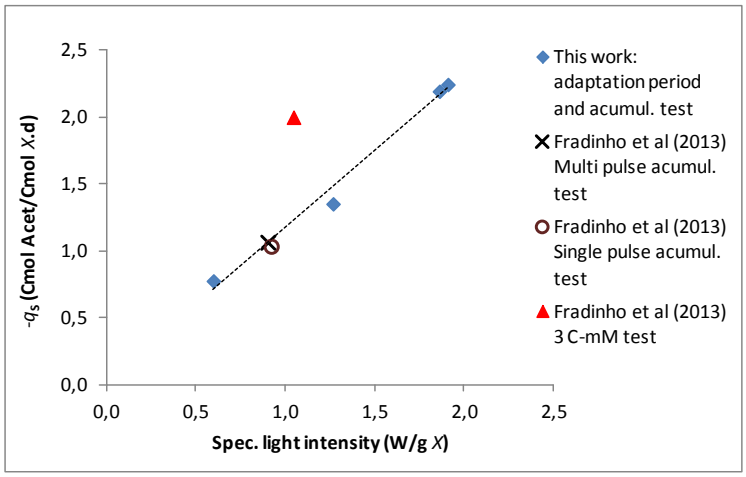

Figure 3.3 – Correlation between the specific light intensity available to the PMC and corresponding specific acetate uptake ($-q_S$) observed during the culture's adaptation period. Indication of Fradinho et. al. (2013) results for comparison.

Comparing the acetate uptake rate obtained in the illuminated phase of this work (day 16) with the PMC acetate uptake rate of Fradinho et al. (2013) at a similar initial acetate concentration of 3 C-mM (Table 3.1-a), it can be observed that similar rates were obtained (2.2 ± 0.17 Cmol Acet/Cmol *X* d and 2.0 ± 0.23 Cmol Acet/Cmol *X* d, respectively). However, the PMC of the present work had a specific light availability (1.9 W/ g *X*) that almost doubled that of Fradinho et al. (2013) (1.05 W/ g *X*). An explanation for the Fradinho et al. (2013) culture presenting a comparable acetate uptake rate at lower light availability is the relatively high carbohydrate consumption rate (0.33 ± 0.16 Cmol Carbs/Cmol *X* d) that was simultaneously occurring, which provided extra energy for substrate uptake. However, in the PMC accumulation tests of Fradinho et al. (2013) (Table 3.1 b-c), where acetate was fed in multiple pulses and only a small carbohydrate consumption was occurring (0.02 to 0.07 Cmol Carbs/Cmol *X* d), light was the only energy source of the culture. Therefore, with 0.91 - 0.93 W/ g *X* of specific light intensity, the culture presented acetate uptake rates of only 1.03 - 1.06 Cmol Acet/Cmol *X* d, agreeing well with the linear trend observed in the present study (Figure 3.3). This finding indicates that

a direct relationship exists between the activity of a photosynthetic culture and the specific light availability, in the absence of other energy sources such as internal carbohydrates.

Throughout the adaptation period, it was also observed an overall increase in the basal PHB levels (Figure 3.1). Over time, the basal PHB levels increased to about 12 to 14 % (g PHB/g VSS), which is significantly higher than the 2 to 5 % observed in Fradinho et al. (2013). This increase could have resulted from the PMC having in the present work, shorter famine phases, with ~3h for PHB consumption, which did not allow complete consumption of this polymer to low levels, while in Fradinho et al. (2013) the famine phase length was 6h and PHB was consumed until nominal levels.

This shorter famine phase may also have led to the decrease in the PMC carbohydrate levels. Despite the fact that the feeding regime promoted the utilization of stored carbohydrate (by feeding in the dark), the short famine phase length did not allow the full conversion of PHB into carbohydrates, resulting in reduced carbohydrates levels.

Nevertheless, the alternating illumination operational strategy applied to the system allowed the selection of a photosynthetic mixed culture that could endure dark periods and can even increase its basal PHB levels in comparison to a continuously illuminated PMC.

3.3.2 PMC microbial characterization

Throughout the culture's adaptation to alternating illumination conditions, changes in the culture's composition were observed. While the culture was initially composed of a consortium of bacteria and algae, with an abundance of both groups, the algal population decreased to residual levels after the adaptation period. Figure 3.4-a1 shows the sludge's initial composition, where small aggregates with a nucleus of algae surrounded by numerous bacteria were commonly observed. After the adaptation period, algae were rarely observed, while the bacterial domain dominated (Figure 3.4-b1). Apparently, algae were more vulnerable in dark periods than bacteria, as they were less able to grow and maintain themselves under the alternating illumination conditions.

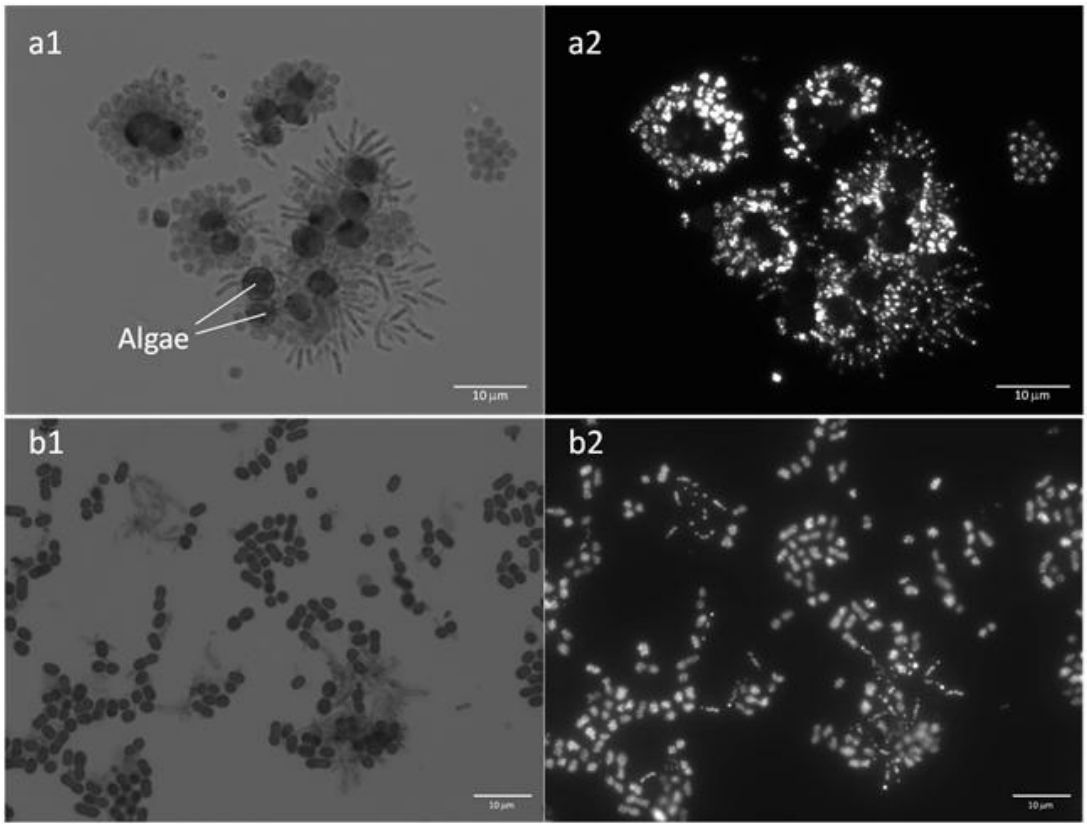

Figure 3.4 – Microscopic images of the PMC initial sludge (a) and after the adaptation period (b). a1, b1 – Bright field; a2, b2 - Fluorescence images of Nile Blue staining indicating PHA granules

Figure 3.4a2 (initial sludge) and b2 (adapted sludge) show through Nile Blue staining the internal PHA granules, where most of the different bacterial morphological groups appeared to accumulate this polymer. Considering the low abundance of algae after adaptation to dark/light cycles, and that algae don't contribute to PHA production, the reduction in algae led to a higher activity of PHA accumulating bacteria and increased PHA levels. This is beneficial towards increasing the PHA accumulation capacity of the culture, and can also simplify the downstream separation of PHA from the biomass.

In relation to the bacterial fraction present in the PMC, FISH analyses indicated a similar prevalence of the bacterial groups present in the starting sludge and in the adapted sludge. Both *Alphaproteobacteria* and *Gammaproteobacteria* groups were present, with the rod shaped bacteria observed in Figure 3.4a1-b1 being identified as *Alphaproteobacteria* and the cocci as *Gammaproteobacteria*. Both groups were observed to accumulate PHA (Figure 3.4a2-b2). All of the other FISH probes that were tested did not show a positive signal.

3.3.3 PMC PHB accumulation capacity

To evaluate the PMC PHA accumulation capacity, an accumulation test was performed with continuous illumination, where acetate was added in small pulses throughout the test to simultaneously prevent carbon depletion and substrate inhibition (Figure 3.5).

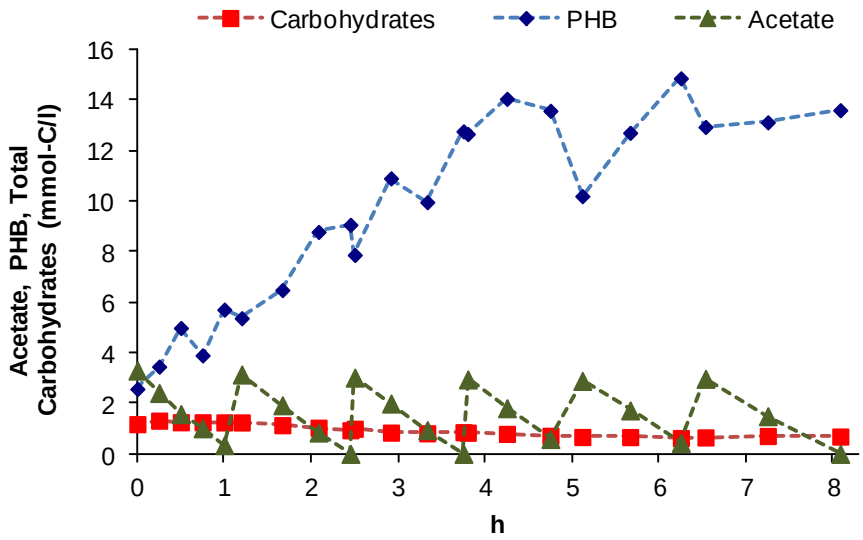

Figure 3.5 – PHB accumulation batch test under constant illuminated conditions with addition of multiple pulses of acetate

Results indicated a continuous PHB accumulation during the first 4 pulses, where a PHB concentration of 14 mmol-C PHB/L was achieved, which corresponded to an accumulation of 30% g PHB/g VSS. The PMC of the present work presented twice the maximum specific PHB production rate obtained by Fradinho et al. (2013), 2.2 ± 0.2 Cmol PHB/ Cmol *X* d and 1.1 ± 0.1 Cmol PHB/ Cmol *X* d in each study, respectively. This higher rate is likely due to the lower number of algae present in the culture, which increases the relative abundance and specific activity of PHA accumulating bacteria, along with the simultaneous increase in light availability, as previously discussed.

Besides the higher maximum specific PHB production rate, the PMC of this study also presented a higher PHB volumetric productivity, in comparison to the PMC enriched under continuous illumination. Though the biomass concentration in Fradinho et al. (2013) was practically the double of the biomass concentration of this work, when the PHB volumetric productivity is calculated, from time zero until a plateau is achieved, a value of 2.7 Cmmol PHB/L h and 1.2 Cmmol PHB/L h is obtained for the PMC of the present work and the PMC studied in Fradinho et al. (2013), respectively. An explanation for this can be that the maximum PHB accumulation rate presented by the PMC in the accumulation test (Figure 3.5) was

maintained constant until a plateau was reached, while in Fradinho et al. (2013), its maximum PHB accumulation rate was only observed in the first pulse, steadily decreasing in the following pulses until stationary PHB levels

In relation to the acetate consumption, it can be observed in Figure 3.5 that a decrease in the acetate uptake rate was observed as the culture became saturated in PHB. In the beginning of the test, the specific acetate uptake rate reached a value of 2.19 ± 0.07 Cmol Acetate/Cmol *X* d, which is very similar to the 2.24 ± 0.17 Cmol Acetate/Cmol *X* d that was observed in the culture at the end of the adaptation period (Table 3.1). As for the PHB accumulation yield per substrate uptake (first 4 pulses), the PMC presented a $Y_{PHB/S}$ of 0.94 ± 0.09 Cmol PHB/Cmol Acet, which translated to 0.90 ± 0.09 Cmol PHB/Cmol of Total Carbon when considering the contribution of both the acetate and carbohydrates towards PHB production. These values are higher than those obtained in the PMC accumulation test (0.70 ± 0.04 Cmol PHB/Cmol Acet) of Fradinho et al. (2013) and accumulation tests with an aerobic mixed microbial system enriched with acetate as the sole carbon source (0.70 to 0.78 Cmol PHB/Cmol Acet) in Serafim et al (2004). The higher $Y_{PHB/S}$ value obtained in the present work may be due to the fact that the culture was exposed to only residual levels of oxygen. Serafim et al. (2004) operated an aerobic system where the organic carbon taken up would also be lost by respiration, and in Fradinho et al. (2013), the oxygen production by algae could also lead to some cell respiration. In the present work, very little oxygen would be present, considering the very low amount of algae present, thus respiration was minimal. Furthermore, algae can also consume acetate (Ogbonna et al., 2000; Perez-Garcia et al., 2011), thus reducing the algal fraction would also lead to higher PHB production yields.

Overall, the application of dark/light periods to the PMC system enabled an increased enrichment in PHA accumulating organisms. The fact that algae were out-competed in a system with reduced illumination periods led to the near-elimination of a microbial group that did not contribute towards PHA production and competed with the PHA accumulating bacteria for light, energy and nutrients. As a result, a higher PHA content, higher $Y_{PHB/S}$ and higher specific PHA productivity were obtained in the PMC, with only half of the illumination energy input required as compared to previous work. The enrichment of a PMC that presents such promising results under alternating illumination conditions opens up the possibility of a system using direct sunlight illumination. For this, future work will focus on the adjustment of the cycle length from 8 to 24h, in order to be comparable to the daily illumination pattern. Also, a cost comparison between the photosynthetic and the aerobic PHA producing systems will be addressed in further studies in order to establish the potential savings of utilizing a PMC for PHA production.

3.4 CONCLUSIONS

A PHA accumulating PMC was enriched in a system with dark/light periods, where a PHA content of 30% was obtained. Moreover, a higher PHB/C yield of 0.90 Cmol/Cmol and twice the specific PHA production rate were achieved in relation to previous works with constant illumination. A direct relationship between light availability and the specific substrate uptake rate was observed, enabling future manipulation of the biomass concentration and illumination intensity in order to further increase PHA productivity. Dark/light periods seem to be a promising means of operating PMCs with lower energetic inputs, minimizing PHA production costs and facilitating direct solar illumination.

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4

Photosynthetic mixed culture polyhydroxyalkanoate (PHA) production from individual and mixed volatile fatty acids (VFAs): substrate preferences and co-substrate uptake

Summary *This work studied the effect of the substrate feeding composition on the polyhydroxyalkanoate (PHA) accumulation capacity of an acetate enriched photosynthetic mixed culture (PMC). From the six tested organic acids – malate, citrate, lactate, acetate, propionate and butyrate – only the last three volatile fatty acids (VFAs) enabled PHA production, with acetate and butyrate leading to polyhydroxybutyrate (PHB) formation and propionate leading to a HB:HV copolymer with a 51% fraction of hydroxyvalerate (HV). Also, results showed an acceleration of butyrate and propionate consumption when fed in the presence of acetate, suggesting that the latter can act as a co-substrate for butyrate and propionate uptake. Furthermore, results suggest that some PMC bacterial groups present a substrate preference for butyrate in relation to acetate and propionate. These findings indicate the possibility of feeding the PMC with cheap VFA rich fermented wastes, leading to a more cost-effective and environmentally sustainable PHA production system.*

The contents of this chapter were adapted from the publication: Fradinho, J.C., Oehmen, A., Reis, M.A.M., 2013. Photosynthetic mixed culture polyhydroxyalkanoate (PHA) production from individual and mixed volatile fatty acids (VFAs): substrate preferences and co-substrate uptake. (Submitted for publication)

4.1 INTRODUCTION

The utilization of plastics by our society has been exponentially increasing in the last decades. Though their presence has unquestionably improved our daily lives, most plastics are produced through the utilization of petroleum-based compounds, a finite source with escalating prices. Also, conventional plastics tend to persist in the environment due to their low biodegradability, leading to an accumulation of solid waste pollution (Khanna and Srivastava 2005). To overcome this, great awareness has arisen in relation to the replacement of traditional plastics by biodegradable bioplastics. Indeed, the production of bioplastics from biorenewable sources has the potential to reduce plastic environmental pollution and contribute to a more sustainable society.

Polyhydroxyalkanoate (PHA), a biopolymer naturally synthesized by many bacteria and intracellularly accumulated as granules, presents characteristics similar to conventional polyolefins making it a promising material for biodegradable plastics production (Laycock et al., 2013).

Currently, PHA based biopolymers are already commercially available, being industrially produced using pure culture systems operated under aseptic conditions and supplied with chemically defined mediums. However, costs associated to these operational conditions increase PHA prices, economically limiting PHA application as a substitute for traditional plastics (Reis et al., 2011). Moreover, PHA production with pure cultures limits the utilization of domestic and agro-industrial wastes. Therefore, efforts have been applied in the utilization of open mixed microbial cultures (MMCs) for a more cost-effective and sustainable PHA production process.

The possibility of operating MMCs for PHA production derives from the ability of bacteria to store PHA in periods of excess external carbon (feast) as carbon and energy reserves. Under carbon starvation (famine), accumulated PHA can be used by bacteria to grow, a feature that determines the survival of PHA accumulating bacteria in relation to non PHA accumulating bacteria. With repeated cycles of feast and famine (FF), the MMC becomes enriched in microbial populations with enhanced PHA accumulating capacity (Reis et al., 2011).

Several systems have then been engineered to select for PHA accumulating MMCs. Whether the system was operated aerobically (Johnson et al., 2009; Albuquerque et al., 2010; Jiang et al., 2012) or in alternating aerobic/anaerobic conditions with glycogen accumulating organisms (GAOs) (Oehmen et al., 2006; Dai et al,. 2007; Bengtsson 2009), studies using chemically defined media and fermented wastes indicated promising PHA accumulation levels when feeding MMCs with volatile fatty acids (VFAs). In particular, Johnson et al. (2009) obtained 89% PHA accumulation in an acetate fed aerobic MMC, a result comparable to pure cultures

values. Also, maximum PHA accumulation values of 75% with fermented sugar cane molasses (Albuquerque et al., 2010) and 77% with fermented wastewater from paper mill effluents (Jiang et al., 2012) have been achieved so far, indicating that different wastes can lead to high and attractive PHA accumulation values that approach MMCs PHA production to an industrial production scale.

Recently, a new MMC PHA producing system was proposed, using a photosynthetic mixed culture (PMC) composed of a consortium of bacteria and algae that unlike aerobic and GAO MMCs, does not require aeration (Fradinho et al., 2013a). The PHA accumulating PMC was selected under illuminated conditions in a FF regime with photosynthetic bacteria accumulating PHA during the feast phase, and consuming it during the famine phase using the oxygen produced by algae. PHA accumulation levels of 20% and 30% PHA/VSS have so far been obtained with PMCs selected under continuous illumination (Fradinho et al., 2013a) and alternating dark/light periods (Fradinho et al. 2013b), respectively. Both studies used acetate as the sole carbon source, with the accumulated PHA being entirely composed of 3-hydroxybutirate (HB) monomers. However, a homopolymer of poly-3-hydroxybutyrate (PHB) is a hard crystalline polymer that leads to stiff and brittle materials (Albuquerque et al., 2011). But, if different monomer units are incorporated in PHA, like 3-hydroxyvalerate (HV), melting temperatures decrease and brittleness is reduced (Arcos-Hernández et al., 2013), originating a copolymer, e.g. P(HB-co-HV), with increased processability and a broader range of applications. Therefore, it is highly desirable to produce PHA polymers with diverse HA monomers.

In aerobic MMCs, substrate feeding composition has been found to be an important parameter that strongly affects PHA monomers diversity (Albuquerque et al., 2011). For instance, studies have shown the formation of copolymers of P(HB-co-HV) with HV up to 89% with propionate as sole carbon source (Jiang et al., 2011). It is then of interest to test if a similar response can be observed when the substrate feeding composition of a photosynthetic mixed culture is altered. Consequently, one of the goals of the present work is precisely to evaluate the capacity of an acetate enriched PMC to use other organic acids as carbon source and determine the impact of different substrates in the culture PHA accumulation capacity and composition. Considering the importance of utilizing agro-industrial residues as feedstock for mixed culture PHA production, the organic acids tested in this work are common products of waste and effluent fermentation (acetate, propionate, butyrate, lactate) or, are organic acids naturally present in grape and fruit industrial wastes (malate, citrate).

Furthermore, aerobic MMCs are usually fed with streams composed of mixtures of these organic acids and preferences for different organic acids have been observed in MMCs fed with

multiple substrates (Albuquerque et al., 2013; Marang et al., 2013). Therefore, the PMC substrate preferences, carbon uptake dynamics and possible synergistic effects when feeding the PMC with mixed organic acids will also be evaluated in this work.

4.2 MATERIALS AND METHODS

4.2.1 Photosynthetic mixed culture operation

The PMC studied in this work was stably operated for 5 months and resulted from subjecting a photosynthetic consortium of bacteria and algae operated with continuous illumination in a feast and famine regime (Fradinho et al., 2013a), to an increase of the carbon source concentration from 3 C-mM acetate in the beginning of the feast phase to 6 C-mM (maintaining the same C/N ratio).

The PMC operation was performed under continuous illumination in a 4.4L sequencing batch reactor (SBR) with 8h cycles, internally illuminated by a halogen lamp (200 W) at a light intensity of 150 W/m^2, which corresponded to a volumetric intensity of 1.3 W/L of culture broth. The SBR was fed with equal amounts of culture medium (122 mL containing per liter: 0.8 g $MgSO_4.7H_2O$, 1.6 g NaCl, 2.2 g NH_4Cl, 0.2 g $CaCl_2.2H_2O$, 16.4 g NaAcetate.$3H_2O$, 20 mL iron citrate solution (1.0g/L), 4 mL trace element solution) and phosphate medium (122 mL containing per liter: 0.13 g KH_2PO_4 and 0.17 g K_2HPO_4), which corresponded to an organic loading rate of 1.3 g COD/L d. At the end of the famine phase of each cycle, 244 mL of the continuously stirred PMC were wasted, resulting in a hydraulic retention time (HRT) and sludge retention time (SRT) of 6 days. Temperature was controlled at 30ºC and argon was continuously sparged (10mL/min) to prevent surface aeration. pH was controlled at 6.5 using 0.5 M HCl.

4.2.2 PMC feeding with individual organic acids

In order to evaluate the possibility of the PMC to utilize diverse organic acids as carbon source and PHA precursors, 6 substrates were tested: acetate (NaAcetate.$3H_2O$), propionate (HPropionate), butyrate (HButyrate), lactate ($Na(_{D,L-})$Lactate), malate ($H(_{L-})$Malate) and citrate (HCitrate.H_2O). The tests were performed using the SBR sludge in a separate 500 mL batch reactor operated under the same conditions of temperature, illumination and pH control, with argon continuously flushing the headspace. During each test, 12 mL of phosphate and 12 mL of culture medium containing the organic acid in test were added to 376 mL of culture broth collected from the SBR at the end of an 8 h cycle (i.e. at the end of the famine phase), making a total volume of 400 mL and an initial organic acid concentration of 6 C-mM. Samples were

collected over a period of 8 to 10h. The batch tests were performed in duplicate for each carbon source, with the exception of acetate that was performed in triplicate.

4.2.3 PMC feeding with VFA mixture

Two batch tests were performed to assess the PMC substrate consumption and PHA production profile when mixtures of acetate, propionate and butyrate were fed to the culture. The tests were conducted as described in 2.2 with the exception that the culture medium fed to the culture did not contain a carbon source.

In the first test, the carbon source was provided by pulse-wise addition of 3 C-M concentrated pulses containing a VFA mixture of acetate, propionate and butyrate at a 4:1:1 C-mol ratio, respectively, leading to pulses with initial organic acid concentration of 6 C-mM.

In the second test, 3 C-M concentrated pulses were also added to the reactor with the exception of the 3rd pulse that was a mixture of only propionate and butyrate (1:1 proportion), and the 5th and 6th pulses that were composed of only butyrate and acetate, respectively.

4.2.4 Analytical Methods

PHA determination was performed by gas chromatography using the method described in Fradinho et al. (2013a). The organic acids concentrations were determined by high-performance liquid chromatography (HPLC) using an IR detector and an Agilent Metacarb 87H column with the exception of citrate, that was determined using an UV detector and a BioRad Aminex HPX-87H column. 0.01 N sulfuric acid was used as eluent with an elution rate of 0.6 mL/min and a 50ºC operating temperature.

Total carbohydrates hydrolysable to glucose were determined using the method described by Lanham et al. (2012) with minor modifications described in Fradinho et al. (2013a).

Volatile suspended solids (VSS) were determined according to Standard Methods (APHA, 1995). The light intensity provided during the tests was measured using a Li-COR light meter LI-250 A equipped with a pyranometer sensor LI-200 SA.

4.2.5 Calculation of kinetic and stoichiometric parameters

The biomass PHA content was calculated as a percentage of VSS on a mass basis ($\%PHA = 100 \times g\ PHA/\ g\ VSS$), where VSS includes active biomass (X), PHA and total carbohydrates. Active biomass was calculated by subtracting PHA and total carbohydrates from VSS.

The maximum specific substrate uptake rate (-q_S in Cmol Subs/Cmol X d), maximum specific PHA production rate (q_P in Cmol PHA/Cmol X d) and maximum specific carbohydrate utilization rate (q_{Carbs} in Cmol Carbs/Cmol X d) were determined by adjusting a linear regression line to the experimental concentrations determined over time and dividing the slope of the fitting at time zero by the concentration of active biomass at that point.

4.3 RESULTS AND DISCUSSION

4.3.1 PMC feeding with individual organic acids

The PMC studied in this work was a consortium of bacteria and algae that had been enriched in PHA accumulating bacteria using a FF strategy. Throughout the enrichment, acetate was the only carbon source provided to the culture and as a result, the accumulated PHA was entirely composed of HB monomers. In order to evaluate if the PMC was capable of consuming other substrates besides acetate and what would be the impact of their utilization in the culture's PHA content, six carbon sources were tested: acetate (for comparison), propionate, butyrate, lactate, malate and citrate. Figure 4.1 shows the results obtained in batch tests where the PMC was individually fed with each substrate.

In Figure 4.1-A, the batch test with acetate, the culture presented a similar behavior to what is typically observed during a SBR cycle in the PMC reactor: during the feast phase (feast-to-famine ratio < 0.13) acetate is consumed, while PHB is formed, and carbohydrates are also consumed as a possible extra energy source for acetate uptake; during the famine phase, PHB is consumed and carbohydrates are restored via PHB oxidation. Furthermore, cells undergo a growth lag phase during the initial hours, as result of an internal growth limitation that arises following a long starvation period. As a result, cell growth is only observed during the famine phase with cells using the internally stored PHB reserves as carbon source.

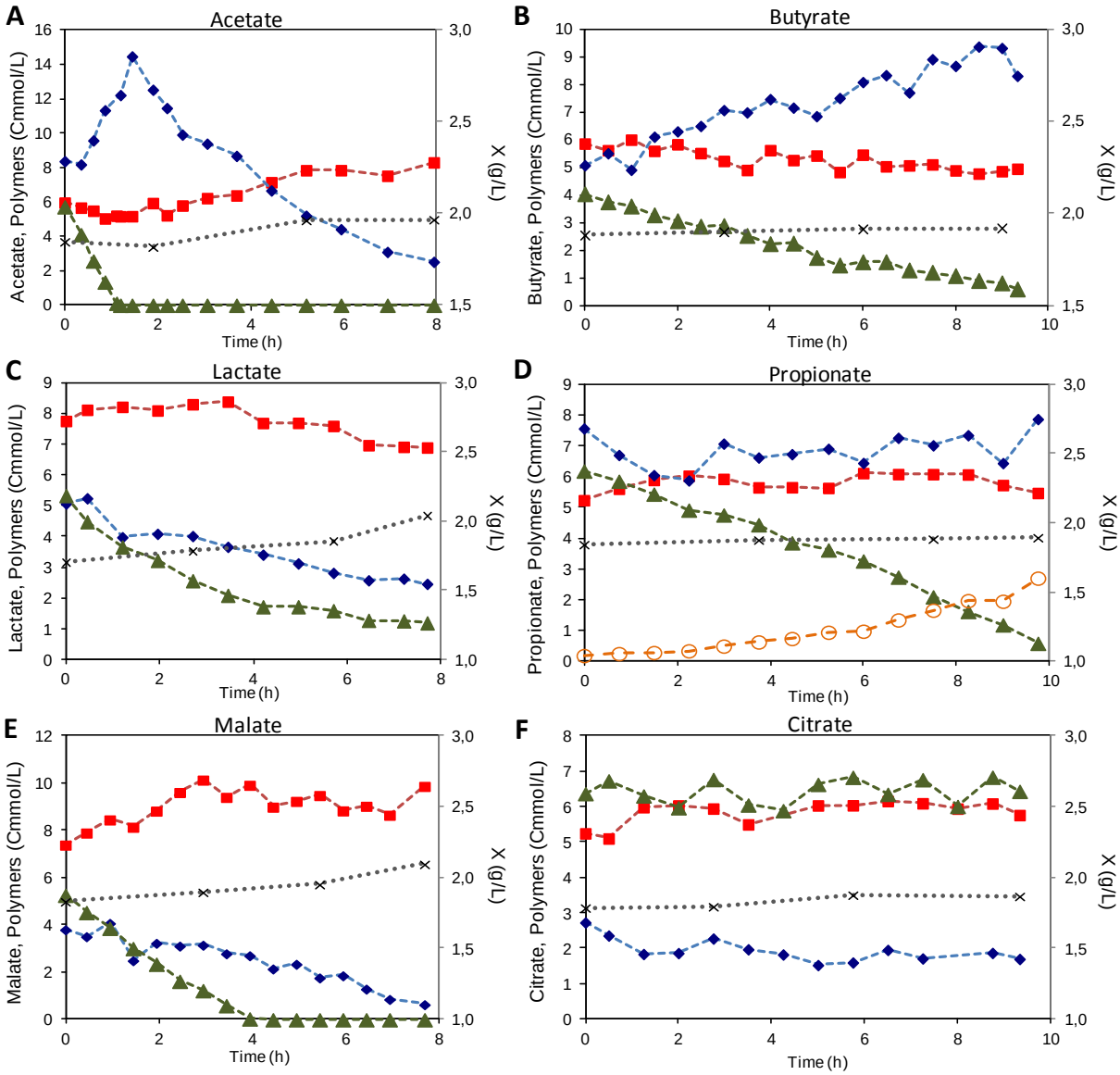

Figure 4.1 – Substrate consumption profile and polymers transformation in batch tests with PMC individual feeding of six organic acids: acetate, propionate, lactate, butyrate, malate and citrate. Organic acid (▲), PHB (◆), PHV (○), carbohydrates (■), *X* - active biomass (×).

From the six tested substrates, acetate was the substrate that was consumed with the highest specific consumption rate, 1.55 Cmol Acet/Cmol *X* d, followed by malate, lactate, propionate, and butyrate (Table 4.1). Citrate, in Figure 4.1-F, was not consumed at all. As an intermediate of the TCA, the absence of citrate consumption does not indicate the inactivity of the PMC tricarboxylic acid cycle (TCA), since the culture can grow on acetate when fed with this substrate, an action that requires the TCA activity. Possibly, PMC organisms lack a citrate transporter for uptake across the cell membrane, and consequently cannot consume it. As a result, cells fed with citrate were in fact exposed to a famine situation and utilized their PHB reserves as carbon source, with some PHB being interconverted to carbohydrates and used for marginal growth.

Table 4.1 – Kinetic and stoichiometric parameters of the batch tests performed with the PMC sludge individually fed with six organic acids: acetate, propionate, lactate, butyrate, malate and citrate. Values in brackets are the standard deviation calculated from two replicate tests.

	q_{PHB}	q_{PHV}	q_{Carbs}	$-q_S$
Acetate	1.50 (0.14)	-	- 0.27 (0.05)	1.55 (0.03)
Propionate	0.07 (0.00)	0.06 (0.04)	-0.02 (0.02)	0.18 (0.01)
Butyrate	0.09 (0.06)	-	- 0.05 (0.01)	0.10 (0.01)
Malate	- 0.23 (0.19)	-	0.30 (0.08)	0.42 (0.03)
Lactate	- 0.15 (0.01)	-	- 0.11 (0.09)	0.28 (0.04)
Citrate	- 0.10 (0.05)	-	0	0

q_{PHB} in Cmol PHB/Cmol *X* d; q_{PHV} in Cmol PHV/Cmol *X* d; q_{Carbs} in Cmol Carbs/Cmol *X* d; $-q_S$ in Cmol Subs/Cmol *X* d

In the case of malate, another TCA intermediate, its substrate consumption rate was the second fastest from all the substrates fed to the PMC with a rate of 0.42 Cmol Malate/Cmol *X* d (Table 4.1). However, its consumption was concomitant with PHB degradation and carbohydrates formation (Figure 4.1-E). Considering its position in the TCA cycle (Figure 4.2), it is likely that PMC cells oxidized malate to oxaloacetate, a gluconeogenesis precursor, leading to the carbohydrates formation observed in Figure 4.1-E. When malate was depleted, carbohydrate production ceased. Acetyl-CoA produced during PHB degradation may have entered the TCA cycle by combination with oxaloacetate obtained from oxidized malate (Figure 4.2). The malate removal via oxaloacetate entrance in the TCA cycle and/or by entrance in the gluconeogenesis pathway may be responsible for the higher malate uptake rate in relation to the other tested substrates, since enzymatic systems for both pathways would already be readily available. As for the cell growth, most of it occurred during the famine phase, in a similar way as it was observed in the acetate batch test.

In the case of lactate, a different substrate consumption profile was observed (Figure 4.1-C). While in the other substrates a linear consumption was observed, lactate consumption rate decreased with time. Since the lactic acid fed in the batch test was an equimolar mixture of $_D$ and $_L$ lactate, it is possible that cells were initially consuming their preferable enanteomeric form of lactate, which most commonly is the D form (Moazeni et al., 2010). This isomeric preference could explain the slower consumption rate of lactate over time.

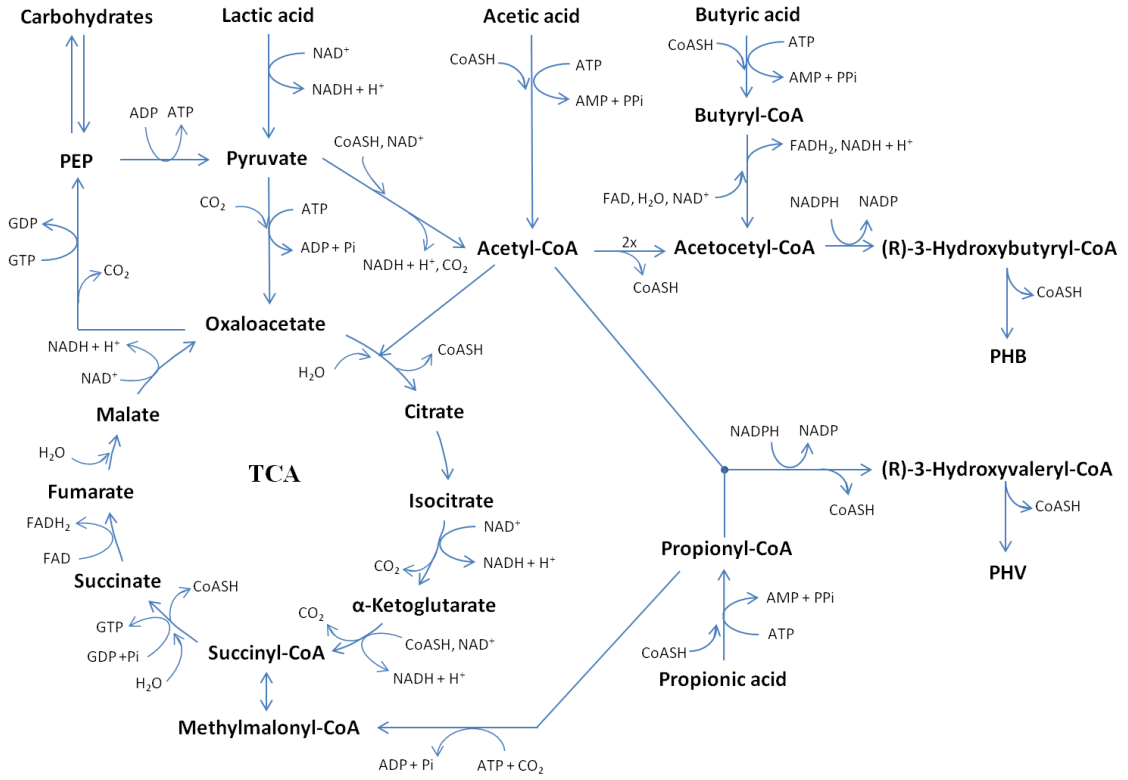

Figure 4.2 – Proposed metabolic pathways of the PMC. (Braunegg et al., 1998; Cooper, 2000; Pisco et al., 2009) TCA – Tricarboxylic acid cycle, PEP – Phosphoenolpyruvate, CoA – Coenzyme A, CoASH – reduced form of CoA.

Considering the lactate feeding impact on the PMC PHA content, Figure 4.1-C indicates a continuous PHB consumption throughout the test. An explanation for this PHB consumption may be found in the cells lactate metabolism. When lactate is fed to the culture, its uptake is likely followed by an oxidation to pyruvate (Figure 4.2). Pyruvate can have multiple fates in cells metabolism: it can be carboxylated to oxaloacetate (and then enter the TCA cycle and/or follow gluconeogenesis), can be transaminated to alanine, decarboxylated to acetaldehyde and further reduced to ethanol, and can also be oxidized and decarboxylated to acetyl-CoA. From all of these pathways, only the last one results in a precursor for PHB production. Since PHB is in a dynamic equilibrium between degradation to acetyl-CoA and formation from acetyl-CoA, if the pathway from pyruvate to acetyl-CoA is not favored in relation to the other pathways, PHB may be consumed for acetyl-CoA replenishment. Apparently, this may be the situation occurring in the PMC. However, other cultures can have organisms that favor the pathway from pyruvate to acetyl-CoA, and therefore, accumulate PHA. Aerobic MMCs enriched with periodic lactate feeding (lactate mixture with propionate and acetate in the work of Dionisi et al. (2004) and only lactate in the work of Jiang et al. (2011)), were shown to be capable of accumulating PHA in tests with lactate fed as single substrate. The acclimatization to lactate during the enrichment period may be the key to select for organisms or pathways that can use lactate for PHA

production. In the present work, the PMC was enriched with acetate and consequently this selection didn't take place. Considering the culture cell growth, it presented a similar behavior to that observed in the case of malate and acetate, with most of the growth occurring after the initial hours, when the culture was in a so-called famine phase: despite some lactate still being present in the medium, the culture was not using it and was therefore, in fact, in a famine situation.

Propionate and butyrate were also tested. These VFAs have been thoroughly studied in aerobic MMC systems as PHA precursors, and in this work, propionate (Figure 4.1-D) and butyrate (Figure 4.1-B) were in fact the only substrates that led to PHA formation. Butyrate enabled the formation of a HB homopolymer, while with propionate, a co-polymer of P(HB-co-HV) was obtained, being achieved a HV molar fraction of 51%. Despite being suitable for PHA production, both substrates presented the lowest specific uptake rates (Table 4.1), with a rate of 0.18 ± 0.01 Cmol Prop/Cmol *X* d for propionate and of just 0.10 ± 0.01 Cmol But/Cmol *X* d for butyrate. Many reasons can be hypothesized for such a difference between the acetate uptake rate and that of the other two VFAs. First, is the culture acclimatization with acetate, where specific enzymes of propionate and butyrate uptake pathways could be sub-expressed or not present at all and therefore lead to lower uptake rates of propionate and butyrate. In fact, a similar result was observed by Lemos et al. (2006) with an aerobic MMC enriched with acetate under a FF regime. This culture also presented inferior substrate uptake rates when instead of acetate, propionate or butyrate was fed.

Further, the butyrate and propionate metabolic pathways can also explain their lower consumption rate. When propionate is taken up, it can be converted to propionyl-CoA and condensate with acetyl-CoA to form PHV. This leads to a demand in acetyl-CoA and reducing power replenishment that can be achieved by propionyl-CoA entrance in the TCA via methylmalonyl-CoA interconvertion to succinyl-CoA (Figure 4.2). The propionate consumption would then be regulated by the rate of its decarboxylation to acetyl-CoA. This can explain its slower consumption rate in relation to acetate fed cells that directly obtain acetyl-CoA from acetate.

In the case of butyrate, extra steps are required for its conversion to PHB or to acetyl-CoA (Figure 4.2). Though its convertion to acetoacetyl-CoA provides enough reducing power for a further conversion to PHB, cells may still require the acetoacetyl-CoA cleavage to acetyl-CoA, since acetyl-CoA is an important precursor for cell metabolism. The higher number of transformations that butyrate must undertake, especially if acetyl-CoA must be produced, may also be the cause for its slower consumption rate.

Interestingly, in both batch tests with propionate and butyrate, practically no cell growth was observed. It could be expected that after surpassing the growth limitation of the initial hours, the culture would grow as observed in the other batch tests. However, this was not the case. Perhaps, when propionate and butyrate were fed to the PMC, the continuous accumulation of the taken up carbon as PHA, combined with the slower metabolism, led to lower resource availability for growth.

Nevertheless, the results with individual substrate feeding demonstrate that an acetate enriched PMC was capable of consuming other substrates besides acetate and utilize two of them, propionate and butyrate, for PHA production. In particular, the PMC could accumulate a copolymer of P(HB-co-HV) when propionate was fed. With these findings it became interesting to test the feeding of a VFA mixture to the PMC, such as is usually present in fermented wastes.

4.3.2 PMC feeding with VFA mixtures

Considering the results obtained with individual substrate feeding, where acetate, propionate and butyrate demonstrated their potential as PHA precursors, a batch test was performed with pulse-wise addition of a solution containing a mixture of those three VFAs in a proportion of 4:1:1 of Acet:Prop:But on a C molar base. The higher amount of acetate in relation to the other two VFAs was considered appropriate taking into account that in the individual feeding tests, acetate presented superior consumption rates, as discussed in Section 4.3.1. It was even expected that with this proportion, acetate could become depleted first. However, results from the batch test with pulse-wise addition of the VFA mixture (Figure 4.3-B) indicated a different outcome. In the first pulse, it can be observed that propionate was in fact the first substrate to become depleted, followed by acetate and butyrate. Comparing the specific uptake rates of each substrate when fed individually with the rates when fed in the mixture, propionate tripled its uptake rate, increasing from 0.18 ± 0.01 Cmol Prop/Cmol *X* d in the individual test to 0.61 ± 0.01 Cmol Prop/Cmol *X* d in the mixture, while the acetate uptake rate decreased nearly by half from 1.55 ± 0.03 Cmol Acet/Cmol *X* d individually to 0.88 ± 0.01 Cmol Acet/Cmol *X* d when mixed with propionate and butyrate. The acetate rate still increased to 1.19 ± 0.01 Cmol Acet/Cmol *X* d, but only when propionate became depleted. As for butyrate, its consumption was practically residual in the beginning, with a rate of just 0.03 ± 0.00 Cmol But/Cmol *X* d. However, when butyrate was the only available substrate, its uptake rate increased to 0.23 ± 0.01 Cmol But/Cmol *X* d. In the individual test it didn't go further than 0.10 ± 0.01 Cmol But/Cmol *X*. These results indicate a significant increase of the propionate and butyrate uptake rate when fed in the presence of acetate.

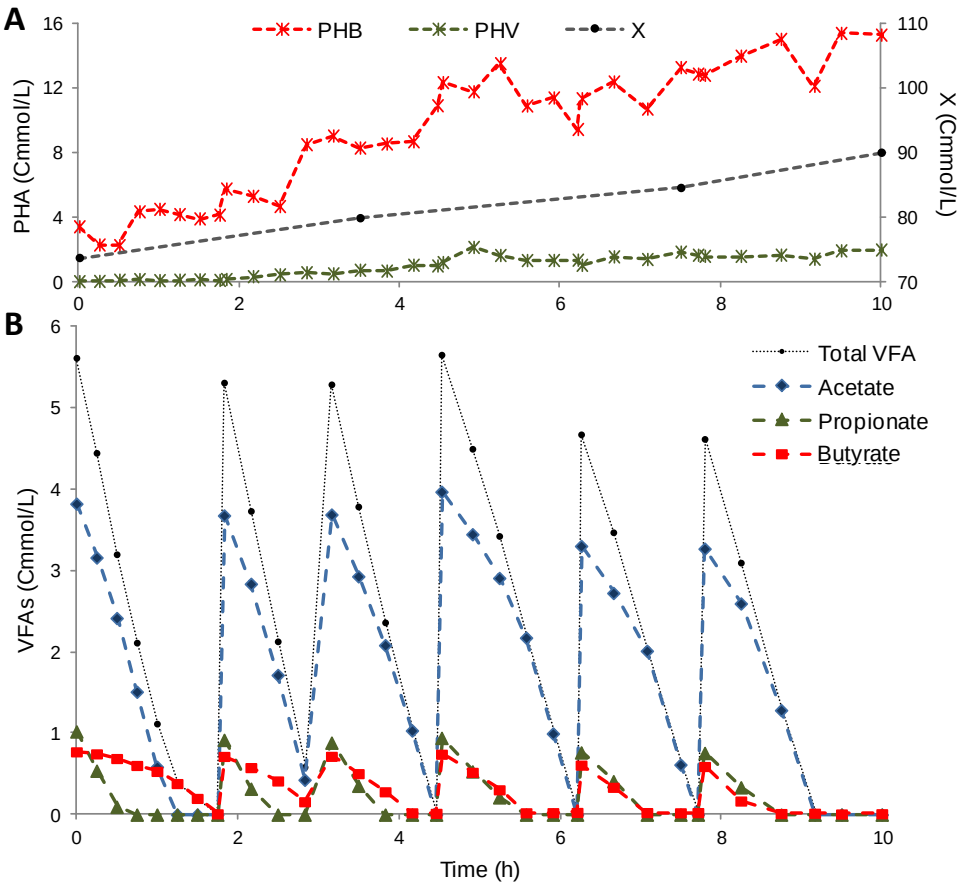

Figure 4.3 – Batch test with pulse-wise addition of a solution containing a mixture of acetate, propionate and butyrate in a proportion of 4:1:1 of Acet:Prop:But on a C molar base. A) PHA accumulation profile, B) VFA consumption profile.

As for the total VFA consumption rate, it remained constant throughout the pulse, with a rate of 1.48 ± 0.04 Cmol VFA/Cmol *X* d, until butyrate was the only substrate left. This total VFA uptake rate is not significantly different than the maximum acetate uptake rate observed in the individual test, 1.55 ± 0.03 Cmol Acet/Cmol *X* d. This shows that the culture tended to split its resources towards acetate, propionate and butyrate, while maintaining a similar efficiency in VFA uptake.

Throughout the pulse additions, the total VFA consumption rate decreased, likely due to the PHA saturation of some organisms as a result of the continuous polymer accumulation. However, a consumption rate decrease was only observed for propionate and acetate along the experiment. On the other hand, butyrate presented an interesting consumption evolution throughout the test. In the first pulse, while propionate and acetate were present, its consumption was rather slow, accelerating after acetate became depleted and when butyrate was the only substrate available. From then on, its consumption rate increased at each pulse addition, even when other substrates were still present in the medium. While acetate and propionate

uptake rates dropped respectively by 50 % and 55 %, from the first pulse to the last one, butyrate tripled its uptake rate. It could be postulated that some organisms present in the culture were synthesizing enzymes for a faster butyrate uptake. This difference in substrate preferences presented by different bacterial groups of a mixed culture has also been observed in aerobic systems. In a mixed culture enriched with fermented wastes containing acetate, propionate, butyrate and valerate, Albuquerque et al. (2013) showed that the culture was composed of different microbial populations specialized in some substrates, with some groups preferring acetate and butyrate while others had broader substrate preferences. In this case, Albuquerque et al. (2013) also observed that the microbial populations' substrate specialization impacted on the substrate removal rates of the different VFAs. Furthermore, even at a species level this could be observed, as reported by Marang et al. (2013) with a single-species dominated mixed culture enriched with acetate and butyrate, which showed an explicit preference for butyrate consumption.

While the PMC was taking up the VFAs, PHA formation was observed. Figure 4.3-A, shows the PHA accumulation profile of this test and it indicates a P(HB-co-HV) co-polymer formation. Fluctuations in the PHA values can be attributed to substrate depletion between pulses that may have resulted in polymer consumption. Therefore, by the end of the test, a plateau had not been reached and the PMC presented a PHA content of 14 %, and formed a co-polymer with an HV molar fraction of 12%. Due to the periodic substrate depletion, maximum specific PHA production rates could not be calculated along the pulse additions, but since the culture was continuously accumulating PHA, an overall value of 0.43 ± 0.03 Cmol PHA/Cmol *X* d was obtained for the entire test. This is significantly lower than the maximum specific production rate of PHB obtained in the individual test with acetate, 1.50 ± 0.14 Cmol PHB/Cmol *X* d (Table 4.1). However, it must be taken into account that in the individual test with acetate, PHA accumulation occurred in the first two hours, a period of internal growth limitation, where most carbon was being direct for storage. On the contrary, the test with pulse addition of a VFA mixture was conducted for ten hours, with cells surpassing the initial growth limitation and using the taken up carbon both for storage and for growth (Figure 4.3), which can also explain the low overall value of PHA accumulation.

4.3.2.1 *Influence of acetate presence in propionate and butyrate consumption rates*

The major outcome from the results of the test with pulse feeding of a VFA mixture is the observation of an increase of propionate and butyrate uptake rates when fed in the presence of acetate. It is not clear, however, how the acetate presence stimulates the propionate and butyrate consumption. As referred in section 4.3.1, when individual substrate feeding was tested,

propionate and butyrate consumption was possibly being limited by an internal acetyl-CoA deficit. Therefore, when fed in a mixture with acetate, its consumption may be facilitated at the metabolic level by the presence of acetyl-CoA that results from the taken up acetate. To clarify this point, a similar test with pulse feeding of the three VFAs was conducted, but with some variations (Figure 4.4).

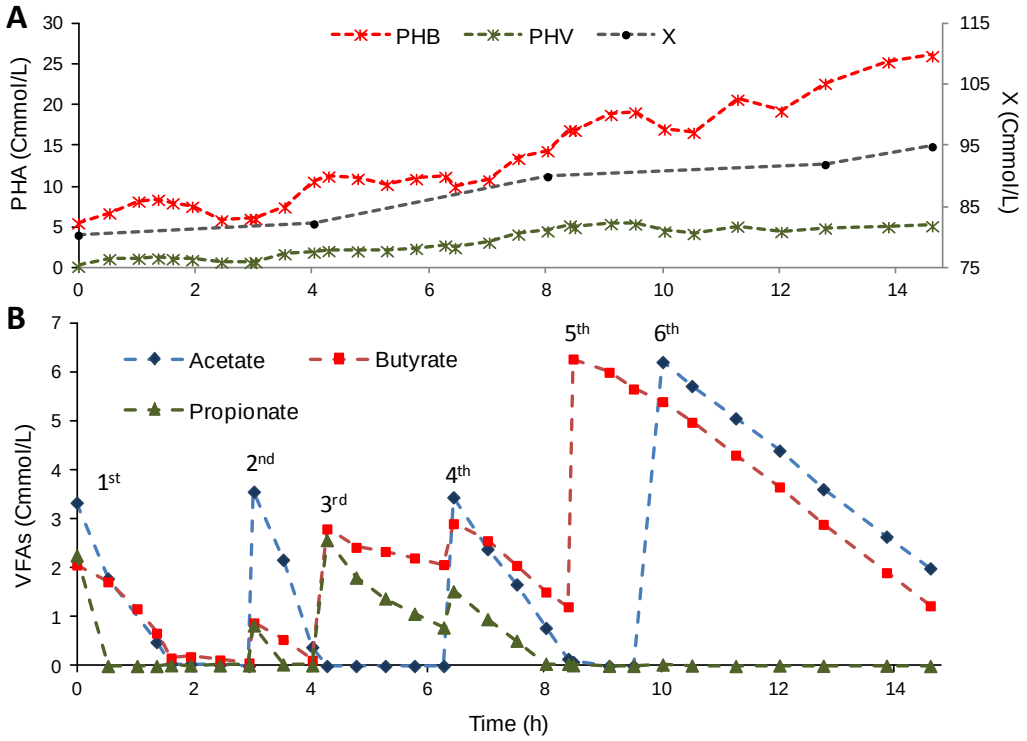

Figure 4.4 – Batch test with pulse-wise addition of a VFA solution containing a mixture of acetate, propionate and butyrate (1st, 2nd and 4th pulses), a mixture of butyrate and propionate (3rd pulse), and only butyrate in the 5th pulse and acetate in the 6th pulse. A) PHA accumulation profile, B) VFA consumption profile.

In the first two pulses, the three VFAs were added together, and as in the previous test, an increase of the propionate and butyrate uptake rate in relation to the individual tests was observed. However, on the 3rd pulse, only propionate and butyrate were fed, and as it can be observed, their uptake rate started to decrease: as more time was passing since the acetate from the 2nd pulse was consumed, more slowly were the propionate and butyrate being taken up. This result excludes the hypothesis that acetate was accelerating the consumption of the other two VFAs by activating the cells metabolism after a famine period. If this was the case, then after two pulses with acetate, the culture would already be active and should have maintained its propionate and butyrate uptake rates. Since this was not the case, it is more likely that acetate is acting as a co-substrate for propionate and butyrate uptake, by increasing cells' metabolic efficiency. As previously referred, the easier access to acetyl-CoA when acetate is consumed

may be facilitating the propionate and butyrate internal processing at a metabolic level. On the 4th pulse, acetate was fed again in the VFA mixture, and immediately, the propionate and butyrate uptake rate increased, corroborating the hypothesis of acetate working as co-substrate for propionate and butyrate consumption.

Furthermore, the importance of acetate being present to accelerate the consumption of other VFA can be observed when comparing the PMC carbon consumption in the 5th and in the 6th pulse. When butyrate was fed alone in the 5th pulse, the culture carbon uptake was just 0.16 ± 0.01 Cmol But/Cmol X d, likely limited by the low acetyl-CoA availability. When acetate was added in the 6th pulse, the culture increased its butyrate uptake rate to 0.25 ± 0.00 Cmol But/Cmol *X* d while was simultaneously taking up acetate at a rate of 0.24 ± 0.00 Cmol Acet/Cmol *X* d. This led to a total carbon uptake rate of 0.49 ± 0.00 Cmol VFA/Cmol X d, which is the triple of the carbon uptake rate when butyrate was fed alone in the 5th pulse. This result indicates the importance of a co-substrate like acetate being present to accelerate other substrates' metabolism, otherwise, much of the culture's carbon uptake potential may be lost.

Concerning the possible culture preference for butyrate, it is interesting that in this test, with the exception of the 3rd pulse, an acceleration of the butyrate consumption rate throughout the pulse additions was also observed. Once again, while acetate and propionate uptake rates decreased along the test, butyrate uptake rate increased. In fact, in the last hours of the test, butyrate consumption rate in a C molar base equaled the acetate one, 0.24 ± 0.00 Cmol Acet/Cmol *X* d, a value similar to the maximum butyrate uptake rate observed in the previous pulsed test.

The PHA accumulation profile was also evaluated in this test (Figure 4.4-A). Once again, a P(HB-co-HV) co-polymer was formed, with HV formation being associated to propionate consumption, and HB formation associated to acetate and butyrate consumption. However, the impact of acetate absence on the VFA consumption profile of propionate and butyrate was reflected on the PHA production profile as well. During the 3rd pulse with only propionate and butyrate present (from the 4th to the 6th hour), PHA production halted, particularly the HB monomers production, where the same occurred in the 5th pulse when only butyrate was available and acetate was absent. This may support the idea that in the absence of acetate, some butyrate might be cleaved to form acetyl-CoA that can enter the TCA and therefore, a lower number of HB monomers can be produced. As for the HV fraction of the PHA, its production ceased when no more propionate was available (~8h), and at that time, the co-polymer presented an HV molar fraction of 24%. After this, only butyrate and acetate were available, and therefore, only HB monomers were produced, decreasing the HV fraction. By the end of the test, the culture presented a PHA content of 22% with a HB:HV molar fraction of 84:16. It is not clear though if higher PHA contents could be achieved since apparently a saturation level

had not yet been reached. Moreover, growth was simultaneously occurring with the PHA production, indicating that part of the taken up carbon was being diverted for cell growth and not used for carbon storage.

Nevertheless, the PHA content attained in this test with pulses of VFAs mixtures is similar to the maximum 20% PHB content obtained by Fradinho et al. (2013a) in an acetate pulsed PHA accumulation test using a PMC also acclimatized to acetate. Though the tests cannot be directly compared, the present work has shown an improvement in relation to the Fradinho et al. (2013a) results, since a P(HB-co-HV) co-polymer was produced while a similar PHA content was attained.

4.3.3 Prospecting PMC enrichment with VFA mixtures

With the knowledge obtained from the tests of this work, future PHA accumulation tests with VFA mixtures may lead to interesting PHA accumulation levels and higher HV fractions. It is now clear that acetate presence is of importance throughout an accumulation test due to its role as a co-substrate in the propionate and butyrate consumption, which is fundamental for an increase of the uptake rates of the latter two. Also, propionate presence is essential if the production of a co-polymer with HV monomers is desired. As for butyrate, its lower uptake rates and subsequent lower PHB production rates would indicate it as a carbon source of minor importance, but this substrate is an important fermentation product that must be considered given its relatively high abundance in VFA mixtures. Also, butyrate is a more energetically favorable substrate since its uptake requires less energy consumption per transported C mol in relation to acetate and propionate (Figure 4.2), which can lead to some organisms having an apparent preference for butyrate.

If the PMC is indeed composed of bacterial groups with different substrate preferences, namely for butyrate, it can be devised that a further enrichment using a mixture of VFAs may lead to a PMC capable of utilizing substrates other than acetate at higher rates. It would be interesting for future work to analyze if changes in the bacterial population will occur and how will they affect the different substrate removal rates. Moreover, changes in the polymer composition will also be expected, since the produced PHA is strongly dependant on the substrate composition and on the culture bacterial species.

Finally, the enrichment of a PMC with mixed VFAs has to be carefully approached because of its unknown impact on the culture algae fraction. Some algae are capable of consuming acetate (Ogbonna et al., 2000; Perez-Garcia et al., 2011) and in an acetate enriched PMC, it is expectable that acetate consuming algae have also been selected as well. Therefore, changing

the substrate composition may also affect the algal population. A reduction in algae biomass would be desirable as long as enough oxygen is produced, enabling the bacterial PHA consumption during the famine phase. In fact, Fradinho et al. (2013b) has shown that diminishing the culture's algae fraction can result in higher PHA contents and higher specific PHA productivities given that algae are a microbial group that does not contribute towards PHA production and competes with PHA accumulating bacteria for light and nutrients. Therefore, enriching a PHA producing PMC under mixed VFA feeding may open up the possibility of using cheap fermented wastes as substrate for PHA production, leading to a more cost-effective and environmentally sustainable PHA production system.

4.4 CONCLUSIONS

The capacity of an acetate enriched PMC to utilize different organic acids for PHA production was evaluated. From the six tested substrates, only acetate and butyrate led to PHB formation, while propionate led to a HB:HV co-polymer formation. Also, an increase of butyrate and propionate uptake rates was observed when both were fed in the presence of acetate. Acetate was likely acting as a co-substrate for butyrate and propionate uptake, by replenishing cells with acetyl-CoA, thus facilitating the internal regulation of cells' metabolism. These results prospect the utilization of VFA-rich fermented wastes as substrate for PHA production in PMC systems.

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5

Beyond feast and famine: selecting a PHA accumulating photosynthetic mixed culture in a permanent feast

Summary *In this work the possibility of utilizing a permanent feast regime as a new operational strategy to select for PHA accumulating photosynthetic bacteria was evaluated. The photosynthetic mixed culture (PMC) was selected in an illuminated environment and acetate was constantly present in the broth to guarantee a feast regime. During steady-state operation, the culture presented low PHA accumulation levels, possibly due to low light availability, which resulted in most of the taken up carbon being used for growth and in a culture growth yield on acetate of 0.64 ± 0.18 Cmol X/Cmol Acet. To confirm the culture light limitation, SBR tests were conducted with higher light availability which resulted in higher PHA contents, being achieved a maximum PHB content of 60%. In this case, growth became minimal and the culture presented a PHB production yield on acetate of 0.67 ± 0.01 Cmol PHB/Cmol Acet. Unlike other studied PMCs, the PMC selected in this work was capable of simultaneously grow and accumulate PHB continuously throughout the cycle. Furthermore, the PMC also presented high phosphate removal rates and allowed culture selection and PHA production in one single reactor, which can lower capital costs. Thus far, 60% PHA content is the maximum value ever reported for a PMC, a result that foresees the utilization of feast regimes as an alternative strategy for selection of PHA accumulating PMCs.*

The contents of this chapter are confidential and are being used for a patent submission.

5.1 INTRODUCTION

Polyhydroxyalkanoates (PHAs) are biodegradable biopolymers that can be synthesized by several microorganisms and internally accumulated as carbon and energy reserves. Because PHAs present physico-chemical properties similar to conventional polyolefins, they have been used in the last years as an alternative material for biodegradable plastics production (Arcos-Hernández et al., 2013; Laycock et al., 2013). However, the currently commercialized PHA is mostly produced using pure culture systems, which contributes to increased PHA production costs due to operation with chemically defined media under aseptic conditions. For this reason, the current PHA market price is not competitive with the traditional plastics one, limiting a wider utilization of PHA based materials (Gurieff and Lant, 2007). As an alternative, efforts have been applied in the utilization of open mixed microbial cultures (MMCs) that enable the utilization of cheap domestic and agro-industrial wastes with no sterility requirements (Reis et al., 2011).

The PHA production process with MMCs commonly requires the selection of a PHA accumulating culture by applying a feast and famine (FF) strategy. This strategy consists in the intermittent feeding of the substrate, where the external carbon is taken up and accumulated intracellularly as PHA (feast phase), followed by phases without substrate addition that favors cell growth on storage products (famine phase), thus creating a selection pressure for organisms capable of storing PHA (Reis et al., 2003). This selection typically occurs in a so-called selector reactor, while the actual PHA accumulation takes place in a second batch reactor where sludge from the selected culture is continuously or pulse fed until cells become saturated in PHA.

Using this strategy, maximum PHA accumulation values of 77% with wastewater from paper mill effluents (Jiang et al., 2012) have been achieved thus far with aerobic MMCs. Recently, a new photosynthetic PHA producing system has been proposed and a feast and famine regime was also used as the selection strategy to enrich a photosynthetic mixed culture (PMC) in PHA accumulating organisms. PHA accumulation levels of 20% and 30% PHA per cell dry weight (cdw) were obtained thus far with PMCs selected using the FF strategy with acetate feeding under continuous illumination (Fradinho et al., 2013a) and alternating dark/light periods (Fradinho et al., 2013b), respectively.

Despite the FF strategy being amply used for the enrichment of aerobic and photosynthetic PHA accumulating cultures with high PHA accumulation capacity, the MMCs PHA production systems require, as previously mentioned, the utilization of two reactors. Moreover, the PHA accumulation step occurs in a batch mode which implies additional time needed for reactor preparation between batch accumulation runs.

As an alternative to the FF strategy, this work proposes a new strategy to select for photosynthetic PHA accumulating bacteria. It consists in maintaining the culture in a feast regime, with permanent presence of external carbon. The selection principle of this feast regime is founded on the unique properties of the anoxygenic photosynthetic bacteria (no oxygen release during photosynthesis). In illuminated environments, these bacteria can use the ATP produced from photosynthesis to take up external carbon. Then, cell metabolism (e.g. growth, polymer consumption) leads to the production of reduced molecules (e.g. NADH, NADPH) that must be oxidized to maintain the cell homeostasis. If no electron acceptors are provided, cells have to activate internal mechanisms to oxidize reduced molecules. One is through the accumulation of PHA that requires its precursors' reduction during the polymer formation. As a result, when photosynthetic bacteria are operated in anaerobic or oxygen limited conditions, only the cells that can dissipate reducing power through PHA production will be able to grow. Therefore, unlike the FF strategy where organisms are selected for their capability of growing on the accumulated PHA, the feast strategy selects organisms for their capability of internally regulating the cell's reducing power through PHA formation. The major advantage of the feast regime in relation to the FF is that its implementation requires only one reactor. This reactor is simultaneously a selector and a PHA accumulator reactor, since organisms are being permanently selected while accumulating PHA. It is expected that the integration of the culture selection step with the PHA accumulation step in a one single bioreactor will lead to a more compact system with subsequent cost reduction and operational simplification.

Therefore, this work studies the possibility of selecting a photosynthetic PHA accumulating culture under a feast regime. Because the feasibility of this strategy implies the observance of several conditions, like the permanent availability of external carbon, the operation under anaerobic or oxygen limited conditions and the availability of excess carbon for PHA storage, these conditions will also be the subject of a thorough evaluation during this study.

5.2 MATERIALS AND METHODS

5.2.1 Photosynthetic mixed culture operation

The inoculum for the PMC studied in this work was obtained from a photosynthetic consortium of bacteria and algae operated in a sequencing batch reactor (SBR) with continuous illumination (1.3 W/L of culture broth) in a feast and famine regime, using acetate as carbon source (Fradinho et al., submitted for publication).

The PMC operation was performed under continuous illumination in a 2.4 L SBR (hence forth called selector SBR) with 24h cycles, externally illuminated by a halogen lamp (60 W) at a light

intensity of 127 W/m^2, which corresponded to a volumetric intensity of 1.8 W/L of culture broth. The SBR was fed with equal amounts of culture medium (400 mL containing per liter: 0.8 g $MgSO_4.7H_2O$, 1.6 g NaCl, 2.2 g NH_4Cl, 0.2 g $CaCl_2.2H_2O$, 16.4 g NaAcetate.$3H_2O$, 20 mL iron citrate solution (1.0g/L), 4 mL trace element solution) and phosphate medium (400 mL containing per liter: 0.13 g KH_2PO_4 and 0.17 g K_2HPO_4), which corresponded to an organic loading rate of 2.3 g COD/L d. At the end of each cycle, 800 mL of the continuously stirred PMC were wasted, resulting in a hydraulic retention time (HRT) and sludge retention time (SRT) of 3 days. Temperature was controlled at 30ºC and argon was continuously sparged (10 mL/min) to prevent surface aeration. pH was controlled at 6.5 using 0.5 M HCl.

5.2.2 PMC operation at higher light availability

In order to evaluate the effect of a higher light availability on the culture performance, two SBRs (450 mL working volume) were operated for 4 days using sludge from the selector SBR. In each SBR, the higher light availability of the culture was achieved by two different ways: by increasing the lamp illumination (SBR 1), or by decreasing the biomass concentration (SBR 2). Therefore, in SBR 1, the reactor illumination was increased to a light intensity of 227 W/m^2, which corresponded to a volumetric intensity of 6.1 W/L (3.4 times higher than in the selector SBR). Also, the SBR 1 test started with the same biomass concentration as in the selector SBR by feeding 75 mL of the culture medium and 75 mL of phosphate medium to 300 mL of culture broth collected from the selector SBR at the end of the cycle. At each 24h cycle, 150 mL of broth were wasted and the reactor was fed with fresh medium. In SBR 2, the reactor illumination was the same as in the selector SBR, but the test started with one third of the selector SBR biomass concentration. This was achieved by centrifuging 300 mL of culture broth and diluting the settled biomass of 100 mL with 300 mL of supernatant. Then, the SBR 2 was fed with 75 mL of the culture medium and 75 mL of phosphate medium and was wasted at each cycle in the same manner as SBR 1. Both SBRs were operated under the same temperature, pH control, HRT and SRT of the selector SBR, with argon continuously sparged. Also, 2 ml pulses of a concentrated solution of acetate and phosphate (4.5 Cmol/L and 0.036 Pmol/L) were added at specific times during the SBRs operation to prevent carbon depletion and to replenish the culture with phosphate.

5.2.3 Analytical methods

PHA determination was performed by gas chromatography using the method described in Fradinho et al. (2013a). Acetate and phosphate concentrations were determined by high-performance liquid chromatography (HPLC) using an IR detector and an Agilent Metacarb 87H

column. 0.01 N sulfuric acid was used as eluent with an elution rate of 0.6 mL/min and a 50ºC operating temperature.

Total carbohydrates hydrolysable to glucose were determined using the method described by Lanham et al. (2012), with minor modifications described in Fradinho et al. (2013a).

Volatile suspended solids (VSS) were determined according to Standard Methods (APHA, 1995). The light intensity provided during the tests was measured using a Li-COR light meter LI-250 A equipped with a pyranometer sensor LI-200 SA.

5.2.4 PMC microbial characterization

Biomass samples were collected throughout the culture enrichment and during the two SBR tests, being examined under the microscope for morphological observation, visualization of intracellular PHA granules (through Nile blue staining) and for bacterial community analysis by fluorescence in situ hybridization (FISH). Nile blue staining was performed on wet biomass according to Bengtsson et al. (2008). For FISH analysis, sludge samples were fixed with paraformaldehyde as described by Nielsen et al. (2009). The following specific oligonucleotide probes were employed: ALF969 for *Alphaproteobacteria* (Oehmen et al., 2006), GAM42a for *Gammaproteobacteria* (details at probeBase: Loy et al. 2007). Each specific probe was applied with a Cy-3 label, together with a FITC-labelled EUBMIX probe for all Bacteria (EUB338 and EUB338-II and III). An Olympus BX51 epifluorescence microscope was used for the microscopic observations of biomass samples.

5.2.5 Calculation of kinetic and stoichiometric parameters

The biomass PHA content was calculated as a percentage of VSS on a mass basis ($\%PHA = 100 \times g\ PHA/\ g\ VSS$), where VSS includes active biomass (X), PHA and total carbohydrates. Active biomass was calculated by subtracting PHA and total carbohydrates from VSS.

The maximum specific substrate uptake rate ($-q_S$ in Cmol Acet/Cmol X d), maximum specific PHA production rate (q_P in Cmol PHA/Cmol X d), maximum specific phosphate consumption rate ($-q_{PO4}$ in mg P/g X d) and specific growth rates (μ in d^{-1}) were determined by adjusting a linear regression line to the experimental concentrations determined over time and dividing the slope of the fitting by the concentration of active biomass at that point.

The yields of PHA per substrate consumed ($Y_{PHA/S}$ in Cmol PHA/ Cmol Acet) was calculated by dividing the amount of PHA formed by the amount of acetate consumed. The yield of growth

per substrate consumed ($Y_{X/S}$ in Cmol *X*/Cmol Acet) was calcu lated by dividing the amount of grown active biomass by the amount of acetate consumed.

The PHB production efficiency (Cmmol PHB/ Wh) was calculated by dividing the PHB productivity (Cmmol PHB/ L h) by the volumetric intensity of the culture broth (W/L).

The specific light intensity of the culture (W/g *X*), was calculated by dividing the volumetric intensity of the culture broth by the active biomass concentration (g *X*/L).

5.3. RESULTS AND DISCUSSION

5.3.1 Photosynthetic mixed culture operation

The operation of the PMC under the new operational strategy of a feast regime resulted in a mixed population of photosynthetic bacteria with decreased algae levels. This diminishment of the algae domain is essential for the feasibility of the feast strategy since it will limit the culture oxygen availability and promote the oxidation of reduced molecules via PHA formation.

During the four months of operation, the culture biomass concentration oscillated (Figure 5.1).

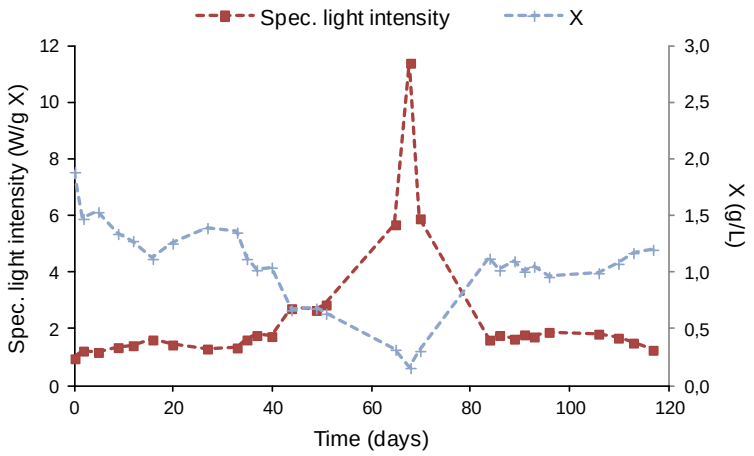

Figure 5.1 – Evolution of the biomass concentration and specific light intensity of the photosynthetic mixed culture during the four months of operation.

In the first 40 days, the culture biomass concentration decreased from 1.9 g *X*/L to 1.0 g *X*/L possibly as an adjustment to the SRT decrease from the 6 days of the seeding sludge SBR to the 3 days SRT of the presently studied culture. Around day 40 and 50, two sequential adverse operational factors occurred (loss of pH control that increased up to 8, then a fused lamp, for 3 days in each case) that in spite of the correct system operation was reestablished, the combination of the two factors led to a massive biomass loss that reached its lower peak at day

67 with a biomass concentration of just 0.16 g *X*/L. After these events, the PMC recovered its biomass levels and maintained a stable biomass concentration around 1.0 – 1.2 g *X*/L for over one month.

The specific light intensity of the culture also varied as a direct consequence of these biomass oscillations (Figure 5.1). During the periods of greater biomass stability, the culture light availability was around 1.2 – 1.9 W/ g *X*. However, it went up to a maximum of 11.4 W/g *X* when the culture biomass concentration was at its lower value. Associated to these biomass and light availability oscillations are also the variations of some nutrients concentrations in the reactor, like acetate and phosphate (Figure 5.2).

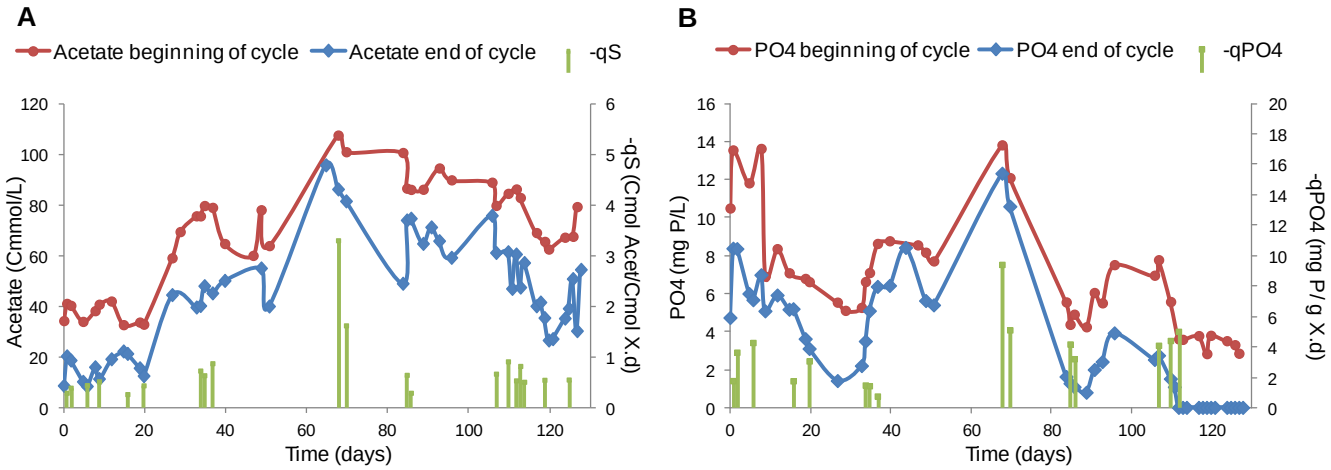

Figure 5.2 – Variation of the acetate (A) and phosphate (B) concentrations in the beginning and end of the cycles during the selector SBR operation. Bars represent the specific substrate uptake rate of acetate (A) and phosphate (B) at the indicated time.

Throughout the SBR operation, it can be seen that acetate was always present in the broth, guaranteeing that the PMC was constantly in a feast regime (Figure 5.2A). While initially the culture was consuming acetate from approximately 40 Cmmol/L in the beginning of each cycle down to 20 Cmmol/L in the end of the cycle, the following biomass loss led to an acetate accumulation in the reactor that achieved concentrations values up to 100 Cmmol/L. When the culture biomass stabilized, the reactor acetate concentration decreased and the SBR cycles were being operated with approximately 70 Cmmol/L of acetate in the beginning of the cycle and 40 – 50 Cmmol/L in the end of the cycle. In the case of phosphate, a similar behavior was observed (Figure 5.2B). However, in the last weeks of the reactor operation, the culture started to consume all the phosphate fed at each cycle, which resulted in a feast and famine regime in relation to phosphate. In spite of this phosphate famine, microscopic observations (data not shown) did not reveal any changes in the PMC microbial population.

Figure 5.2 indicates another interesting fact which is the acetate and the phosphate specific uptake rates achieving maximum values during the lower biomass concentration period (around day 67). It could be thought that during this period, cells would be under a harsh recovering process, but apparently, the surviving cells were quite active taking up acetate and phosphate. Possibly, the few surviving cells were benefiting from the momentary higher light availability that enabled the production of larger amounts of ATP that could be used in the cell's metabolism. A similar occurrence was also observed by Fradinho et al. (2013b) with a PMC operated in dark/light cycles, where reduced illuminated periods resulted in a biomass concentration decrease which led to a higher light availability of the culture and higher specific substrate uptake rates. In the present work, the biomass concentration decrease and the higher light availability apparently also impacted in the cells PHA accumulation profile.

Figure 5.3 shows the cells polymer content evolution during the PMC operation.

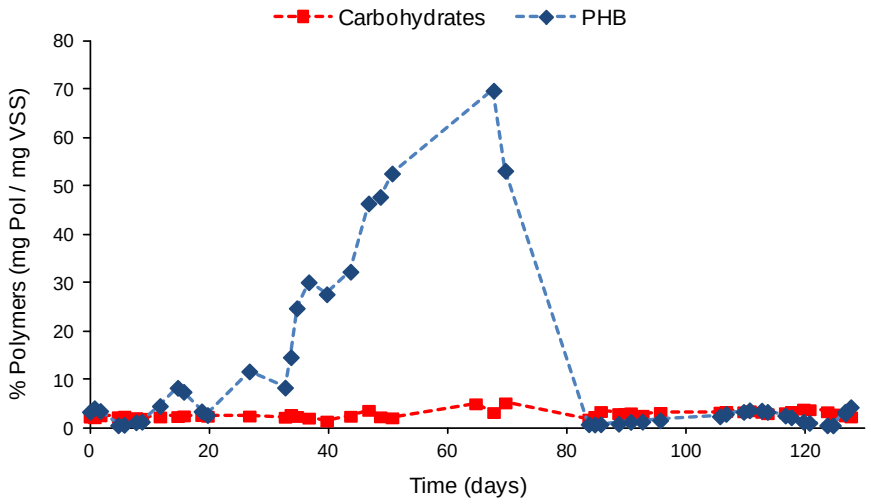

Figure 5.3 – Evolution of the PMC polymer content (PHB and carbohydrates) during the PMC operation

It can be observed that the PHA storage increased radically in the period when the PMC biomass concentration was lower and the specific light availability was higher, being attained a maximum PHA content of 70%. This higher PHA content was corroborated by microscopic observations of Nile blue stained biomass samples collected at that time (Figure 5.4B).

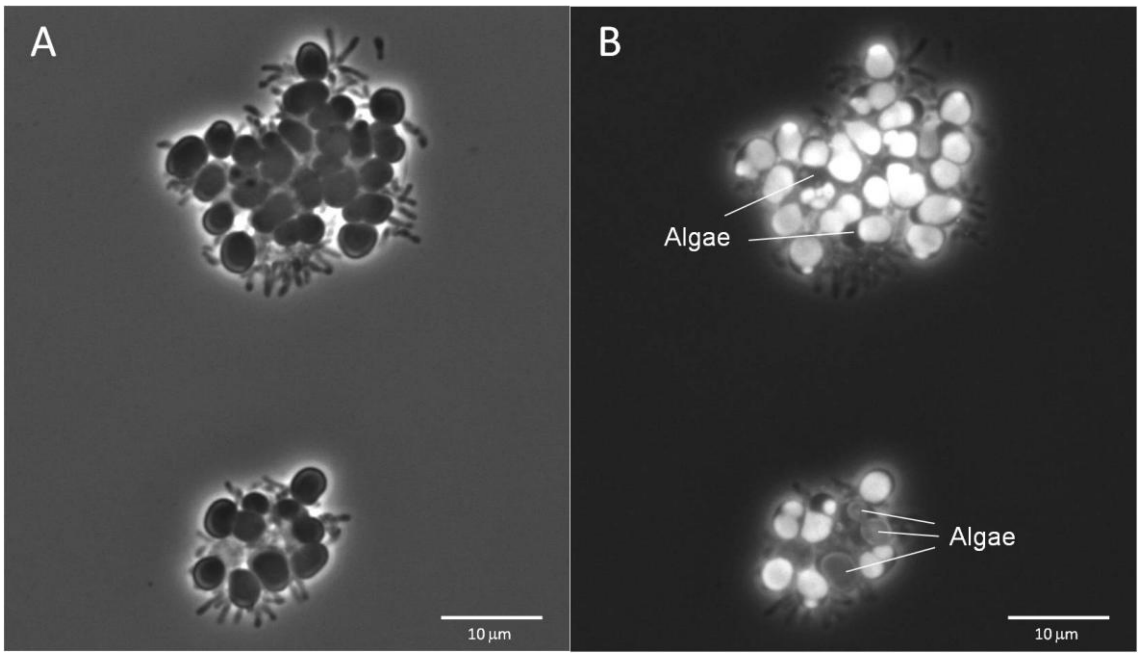

Figure 5.4 – Microscopic images of the PMC of the selector SBR taken during operation at the low biomass concentration. A – Bright field; B - Fluorescence images of Nile blue staining indicating PHA granules.

According to Figure 5.4B, some organisms may have achieved even higher PHA contents, since the PHA inclusions of some cells seem to occupy the majority of the cell and because apparently, not all of the PMC organisms were accumulating PHA (like some of the observed rod shaped bacteria). Nevertheless, when the culture recovered its biomass concentration, the PHA levels decreased down to 3 – 5 %, a similar content to that presented before the biomass loss.

As for the PMC carbohydrate content, despite a minor increase during the low biomass concentration period, it presented low levels around 3 – 5 % throughout the whole PMC operation (Figure 5.3). These polymer results indicate that when the culture was being stably operated, cells were not internally storing the taken up acetate, neither as PHA nor as carbohydrates. To clarify which events were then occurring during the stable PMC operation, several SBR cycles were followed and the typical culture behavior can be seen in Figure 5.5.

During a cycle of the selector SBR (Figure 5.5A), it can be observed that acetate was linearly consumed, but was not exhausted, maintaining the PMC in a feast regime. The average value of the acetate uptake rate from five selector SBR cycles was 0.69 ± 0.08 Cmol Acet/Cmol *X* d (Table 5.1). This value is inferior to the acetate uptake rate of a PMC also selected with continuous illumination but in a feast and famine regime which presented a value of 2.0 ± 0.23 Cmol Acet/Cmol *X* d (Fradinho et al. 2013a).

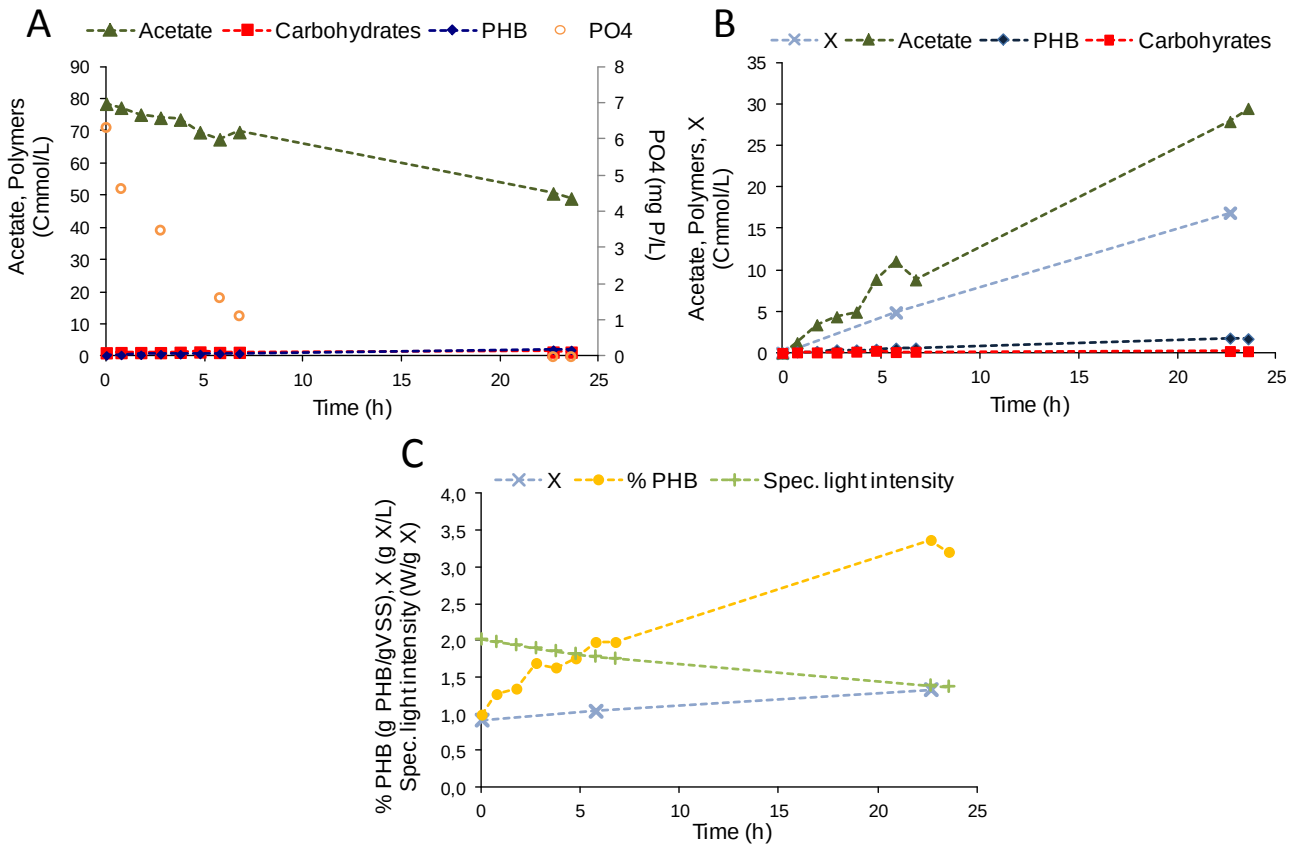

Figure 5.5 – Culture performance during a 24h cycle of the selector SBR conducted during the stable operational period. A – Acetate and phosphate consumption profile and polymers transformation. B – Cumulative values of consumed acetate, produced PHB, produced carbohydrates and produced biomass. C – Variation of biomass concentration, specific light intensity and cells' PHB content.

However, it must be referred that feast and famine regimes promote high specific substrates uptake rates by selecting fast carbon consuming and PHA accumulating organisms, but that these high carbon consumption rates only occur for few hours due to the short period of the feast phases. Overall, the PMC of the present work presents a higher global carbon removal since it is operated in a feast regime, and therefore, it is continuously consuming acetate during the entire cycle.

Unlike acetate, phosphate was totally removed, and the time at which it would become depleted varied from 5 to 10 h, depending on the analyzed cycle. PHA had marginal production and this can be explained by observation of Figure 5.5B, where most of the consumed acetate was used for cell growth, with the PMC presenting growth yields on acetate around 0.64 ± 0.18 Cmol *X*/Cmol Acet and only 0.07 ± 0.05 Cmol PHB/Cmol Acet for the PHA production yield per acetate consumed (Table 5.1). As a result, the culture PHA content did not suffer great variation, just slightly increasing from 1 to 3 – 5 % (Figure 5.5C). In the case of carbohydrates, no variations were observed in their content.

Table 5.1 – Kinetic and stoichiometric parameters of the PMCs performances of the selector SBR, of SBR 1 and of SBR 2.

	q_P	$-q_S$	$-q_{PO4}$	$Y_{PHB/S}$	$Y_{X/S}$	μ
Selector SBR[a]	0.05 (0.04)	0.69 (0.08)	17.4 (2.92)	0.07 (0.05)	0.64 (0.18)	0.51 (0.13)
SBR 1	0.73[b] (0.13)	1.49[b] (0.33)	52.7[b] (40.1)	0.67[c] (0.01)	0.11[c] (0.01)	0.19[b] (0.11)
SBR 2	0.23[b] (0.18)	1.69[b] (0.15)	52.4[b] (11.7)	0.24[c] (0.01)	0.35[c] (0.01)	0.82[b] (0.15)

q_P in Cmol PHB/Cmol *X* d; $-q_S$ in Cmol Acet/Cmol *X* d; $-q_{PO4}$ in mg P/g *X* d; $Y_{PHB/S}$ in Cmol PHB/Cmol Acet; $Y_{X/S}$ in Cmol *X*/Cmol Acet; μ in d^{-1}

[a] Average values calculated from 5 SBR cycles

[b] Values are the average specific rates calculated for the first 3 days of the test

[c] Values were calculated for the first 3 days of the test

Overall, the implementation of a new system using a feast regime to select for PHA accumulating photosynthetic organisms led to a PMC that presented low polymer content when stably operated at biomass concentrations of 1.0 – 1.2 g *X*/L and specific light intensities around 1.2 – 1.9 W/g *X*. However, the 70 % PHA content that the culture presented when it had a low biomass concentration and a high light availability, suggest that during the stable periods, the culture was possibly being operated under light limitation, and consequently, had low energy availability. As a result, the culture growth requirements would absorb practically all the taken up acetate, decreasing the carbon availability for polymer storage. If the culture was in fact under light limitation, it is possible that an operational adjustment to higher light availabilities could lead again to the higher PHA contents previously observed.

5.3.2 PMC operation at higher light availability

To evaluate the hypothesis that with the PMC operation at higher light availability the culture can achieve high PHA contents, two tests were conducted for 4 days in two separate SBRs, using sludge from the selector SBR. In SBR 1 the higher light availability was achieved by increasing the reactor illumination, with the test starting with the same biomass concentration of selector SBR when stably operated (~1.1 g *X*/L), which resulted in an initial specific light intensity of 5.6 W/g *X* (over 3 times higher than in the selector SBR). In SBR 2, the higher light availability was achieved by decreasing the biomass concentration by one third (0.4 g *X*/L), maintaining the reactor illumination equal to the selector SBR one, which resulted in an initial specific light intensity of 3.5 W/g X (over 2 times higher than in the selector SBR). Results can be observed in Figure 5.6.

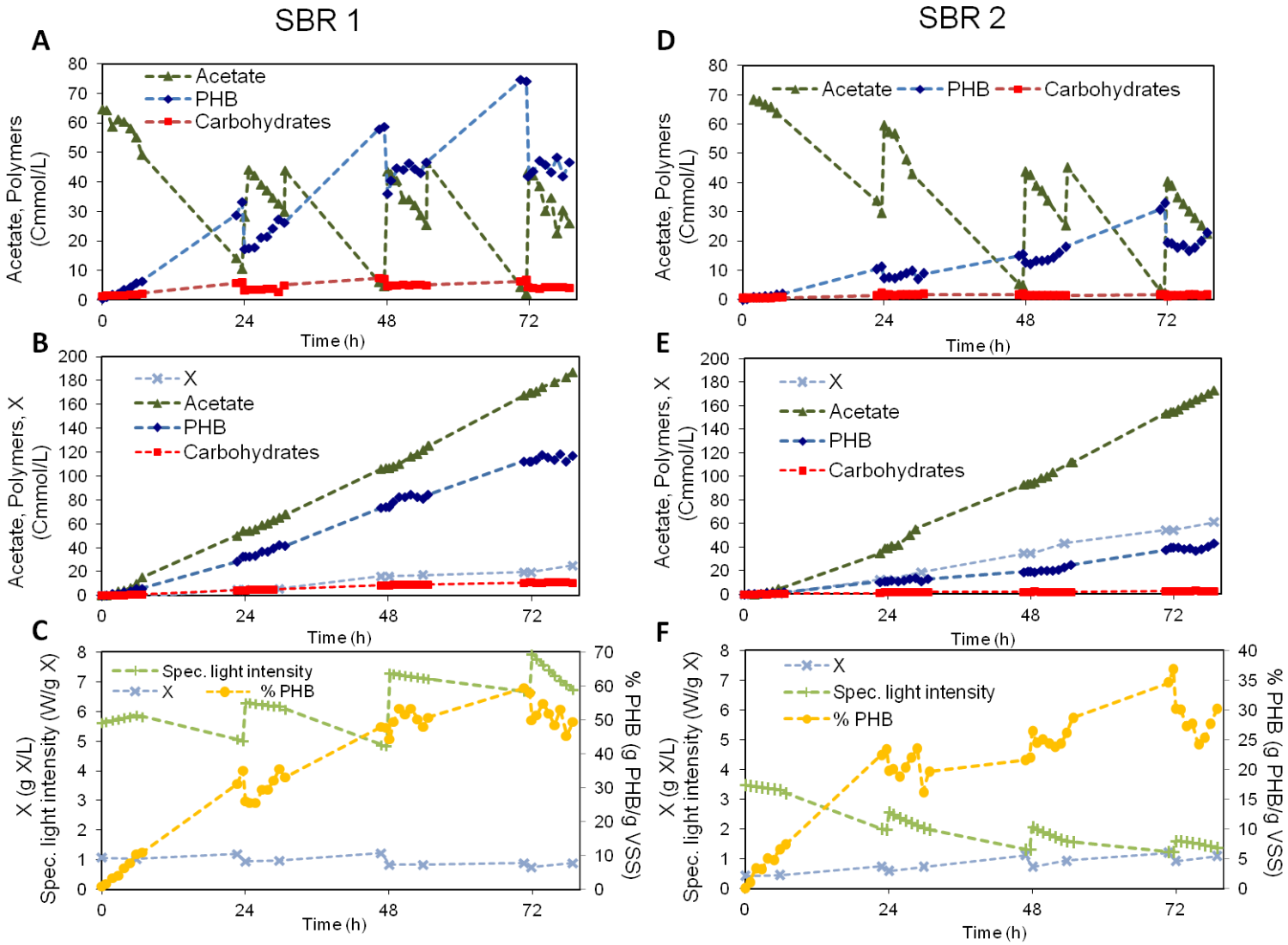

Figure 5.6 – Overall operational profile of SBR 1 and SBR 2 during the 4 days test. A and D – Acetate consumption profile and polymers transformation. The reactor's waste is reflected in the decrease of PHB and carbohydrate concentration; the addition of fresh medium is reflected in the increase of acetate concentration. B and E - Cummulative values of consumed acetate, produced PHB, produced carbohydrates and produced biomass. C and F – Variation of biomass concentration, specific light intensity and cells' PHB content.

During the SBR 1 operation (Figures 5.6A-C), the acetate consumption and polymer production (Figure 5.6A) was superior to that observed in the selector SBR (Figure 5.5A), with a two times higher specific acetate consumption rate and almost 15 times higher specific PHA production rate (Table 5.1). Because of this such higher acetate consumption rate, a pulse of acetate was added in the second and third days of operation to prevent carbon depletion and maintain the feast regime. A similar situation occurred in the SBR 2 (Figure 5.6D), with a pulse addition on the third day. Interestingly, the acetate uptake rate in SBR 2 also doubled that of the selector SBR, but the PHB production rate was only five times higher (Table 5.1).

From Figure 5.6B, it can be seen that most of the taken up acetate in SBR 1 was accumulated as PHB, with few carbon being used for growth. On the contrary, in SBR 2 more acetate was used for growth than for storage (Figure 5.6E). This different usage of the taken up acetate became reflected in the cultures yields, with SBR 1 presenting a PHB production yield on acetate of 0.67 ± 0.01 Cmol PHB/Cmol Acet and SBR 2 presenting only 0.24 ± 0.01 Cmol PHB/Cmol Acet (Table 5.1). As a result, after three days of reactor operation, the SBR 1 culture presented a PHB content of 60% (Figure 5.6C), while in SBR 2, only a 35% PHB content was achieved (Figure 5.6F). Once more, the different PHB content of each culture could be observed by Nile blue staining (Figure 5.7).

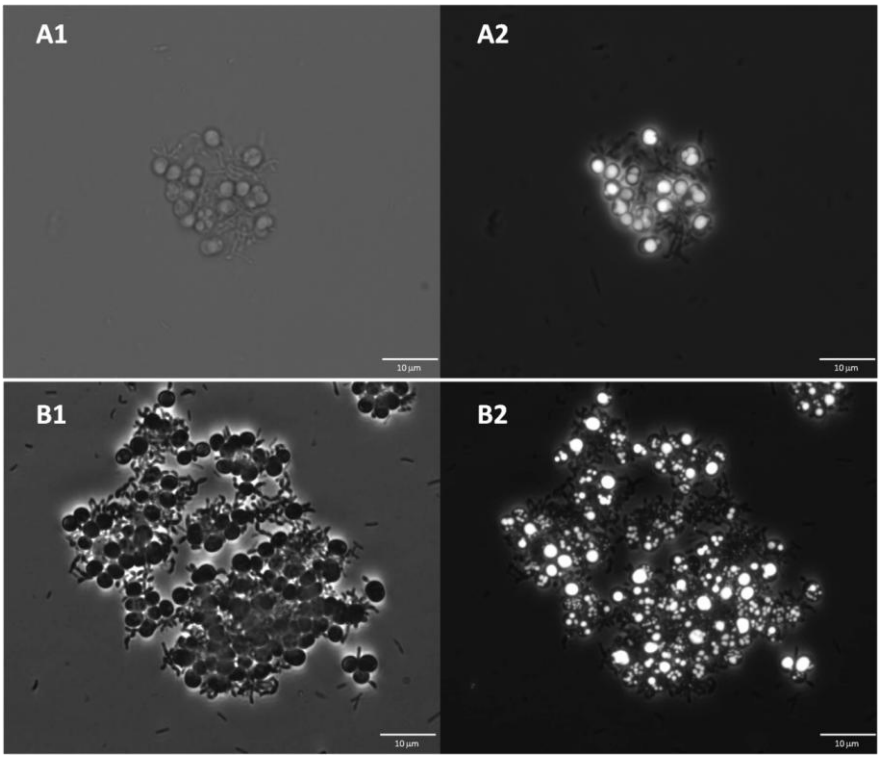

Figure 5.7 - Microscopic images of the PMC of SBR 1 (A) and SBR 2 (B). A1 and B1– Bright field. A2 and B2 – Fluorescence images of Nile blue staining indicating PHB granules.

In Figure 5.7.A2 it can be seen that the SBR 1 cocci shaped bacteria were mostly full with polymer while in SBR 2 (Figure 5.7.B2), some were full as well but many more had just some PHB granules, which agrees with the lower measured PHB content. In both SBRs, the rod shaped bacteria appeared to not accumulate PHB, like what was observed for the selector SBR bacteria.

To find an explanation for these different PHB accumulation values between the two SBR cultures it was hypothesized that a nutrient limitation could be occurring in SBR 1 that would holdup growth and divert the taken up carbon for storage. However, ammonia was constantly present in both SBRs (data not shown) and therefore, it was not limiting growth. On the other hand, phosphate depletion was occurring in both SBRs (Figure 5.8).

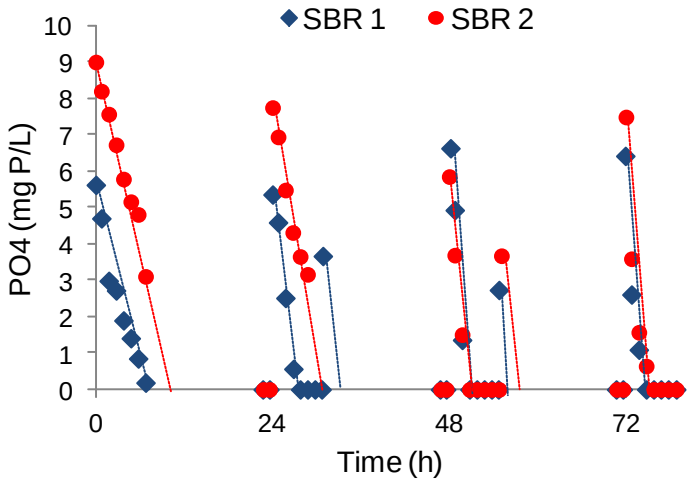

Figure 5.8 – Variation of the phosphate concentration in SBR 1 and SBR 2 during the 4 days test. Dotted lines facilitate the visualization of the phosphate consumption profile.

However, phosphate depletion could not explain the limited growth and higher PHB accumulation of SBR 1, because if this was the case, then the selector SBR should have also had high PHB content when it was being stably operated during phosphate depletion (Figure 5.5A) and should have had low PHB content during the biomass loss period because there was more phosphorous available for growth (Figure 5.2B).

Then, another explanation for the different PHB contents could be the different specific light availability of both cultures. SBR 1 culture started with a higher specific light availability than the SBR 2 culture, a light availability that even increased throughout the test as a result of the reduced growth and consequent decrease of the biomass concentration (from 1.1 g *X*/L to 0.8 g *X*/L) (Figure 5..6C). With more available light, more energy could be used for acetate uptake

and carbon storage, as previously explained. On the other hand, SBR 2 started with a specific light intensity that was higher than the selector SBR one (which explains its higher PHB content in relation to the selector SBR culture content during stable operation), but that was lower than that in SBR 1. Therefore, the SBR 2 culture would have less energy available to accumulate acetate as PHB. However, the specific acetate uptake rate of the SBR 2 culture was similar to the specific acetate uptake rate of the SBR 1 culture (Table 5.1). So, the SBR 2 culture had in fact carbon available for storage, but instead, growth was promoted. The specific growth rate of this culture (0.82 ± 0.15 d^{-1}) was also superior to that of the selector SBR growth rate (0.51 ± 0.13 d^{-1}). So it is then questioning what could make the less concentrated culture have a faster acetate uptake rate than that expected (considering the available light) and have a growth even superior than that of the selector SBR culture. Future work will focus on this question, but thus far it can only be speculated that since the SBR 2 started with a biomass dilution, quorum sensing could have had an effect on cells metabolism. Also, it cannot be discarded the hypothesis that the higher PHB production of SBR 1 cells could also have been stimulated as a protection response to the strong illumination, since PHA granules may offer protection against UV light irradiation (Castro-Sowinsk et al. 2010). Perhaps several factors may have influenced the different PHA contents of the two SBRs.

Nevertheless, and despite the fact that SBR 2 presented higher cell growth and lower carbon storage in relation to SBR 1, these two tests confirm that operating the PMC with higher light availability, can lead to higher PHA contents and enable the operation of a PHA accumulating culture in a system with a permanent feast regime.

5.3.3 Microbial characterization

The initial sludge that was used to seed the SBR of this work was originally composed of a consortium of bacteria and algae, where the bacterial domain was mostly represented by *Alphaproteobacteria* and *Gammaproteobacteria*, similarly to what was observed in other PMC cultures (Fradinho et al. 2013a, 2013b). Considering the algae domain, a decrease in the population of these organisms was observed. However, the size of each algae cell (~7.2 μm) was higher than that observed in algae of other PMC cultures (~3.7 μm). This occurred both in the selector SBR (Appendix Figure A and B) and in the two SBRs tested with higher light availabilities (Appendix Figure C and D). It is not clear though if this higher cell size resulted from an adaption of previous algae species to the new operational conditions or if these are new algae species.

In relation to the bacterial fraction present in the PMC, FISH analyses indicate once again the presence of *Alphaproteobacteria* (most of the rod bacteria) and *Gammaproteobacteria* (cocci shaped bacteria). However, the *Alphaproteobacteria* of the present culture apparently do not accumulate PHA, unlike what was observed in the *Alphaproteobacteria* from previously studied PMCs. This can be an indication that the species composition of the *Alphaproteobacteria* organisms may have changed as well. Considering the *Gammaproteobacteria* fraction of the present culture, Nile blue staining indicated that this was the main bacterial group responsible for PHA accumulation. Morphological observations showed that these cocci shaped organisms present a bigger cell size (~3.2 μm) than the *Gammaproteobacteria* of the previously studied PMC cultures (~1.4 μm). This bigger cell size occurred even in situations where the cell PHB content was low, so internally stored polymers were not affecting the organisms' morphology. Another important characteristic of the presently studied *Gammaproteobacteria* is the occasional appearance of vacuoles, a feature that had not been previously observed in the *Gammaproteobacteria* of other PMCs. All of this evidence indicates that the new operational conditions tested in this work, namely the reduction of SRT to 3 days and a continuous feast regime, may have led to possible alterations in the culture's species composition, both in the algae and in the bacterial domain. To clarify these microbial alterations, advanced molecular techniques, like pyrosequencing, will be used in future tests, thus enabling the identification of the species that contribute to the PMCs composition.

5.3.4 Feast and Famine vs Feast

In this work, a new system was proposed to select for photosynthetic PHA accumulating bacteria using a permanent feast strategy. In previous studies, feast and famine strategies were used to select PMCs capable of accumulating PHA (Fradinho et al., 2013a, 2013b), following a practice that is commonly used in aerobic mixed microbial cultures. Both strategies led to the selection of photosynthetic PHA accumulating bacteria, and because both have advantages and drawbacks, a comparison of the results obtained so far was performed.

First, it is important to compare the PHA productivity obtained with both systems. Table 5.2 summarizes the results obtained with the two SBRs tested in the present work, and the PHA accumulation tests conducted with PMCs enriched under feast and famine conditions under continuous illumination (Fradinho et al., 2013a) and under alternating illumination conditions (Fradinho et al., 2013b).

Table 5.2 – Volumetric intensity, maximum PHB productivity, maximum PHB production efficiency and PHB content obtained with PMCs operated in SBR 1 and SBR 2 of the present work and obtained in accumulation tests with PMCs selected in feast and famine regimes under continuous illumination (Fradinho et al., 2013a) and under alternating illumination conditions (Fradinho et al., 2013b).

	Feeding strategy; Illumination	Volumetric intensity (W/L)	Maximum PHB productivity (Cmmol PHB/L h)	Maximum PHB production efficiency (Cmmol PHB/Wh)	PHB content (%)
SBR 1	Feast; Continuous	6.1	1.6	0.26	60
SBR 2	Feast; Continuous	1.5	0.53	0.36	35
Accumulation test Fradinho et al. (2013a)	Feast & Famine; Continuous	1.3	1.2	0.94	20
Accumulation test Fradinho et al. (2013b)	Feast & Famine; Dark/Light	1.3	2.7	2.0	30

It can be observed that the highest productivity, 2.7 Cmmol PHB/L h, was obtained when operating a PMC under feast and famine and dark/light periods. This productivity was obtained with just 1.3 W/L of illumination, making this system the one with higher PHB production efficiency, i.e., with more polymer being produced per energy unit applied. This higher efficiency likely resulted from the fact that this culture was not only selected under a feast and famine of carbon, but also, under a feast and famine of light, which enabled the selection of more efficient bacteria with respect to light utilization. The PMC operated in the SBR 1 of this study presented the second highest productivity, but it had 4 times more light availability than the other systems, thus presenting the smallest PHB production efficiency. Close to the SBR 1 PHB productivity is the PMC operated in a feast and famine regime with continuous illumination, followed by SBR 2. However, these last two presented a higher PHB production efficiency than SBR 1. In the case of SBR 2, because its culture's higher light availability was achieved by reduction of biomass concentration, a low light input was provided (1.5 W/L) and despite the substantial utilization of consumed carbon for growth, a higher polymer production efficiency was obtained in relation to SBR 1.

The results obtained with the different studied PMCs indicate that the operation in a FF regime and dark/light periods is the best option, so far, to select for PMC with an increased PHB productivity and production efficiency. But because the feast regime has not been operated yet under transient illumination conditions, it can only be directly compared with the feast and famine regime operated also in similar conditions, i.e. in continuous illumination. Consequently,

when comparing both systems, the PMC of SBR 1 presents a higher PHB productivity than the PMC of Fradinho et al., 2013a, but lower production efficiency.

From an economical point of view, if artificial illumination was to be used, then the SBR 1 system would be unfavorable due to the higher energy requirements for PHB production. However, the main goal of operating photosynthetic cultures is to utilize direct sunlight illumination, which is a free energy source that can provide high illumination inputs (sunlight can illuminate the Earth surface with an irradiation of 1360 W/m^2 (Kopp and Lean 2011), six times higher than the irradiation provided to SBR 1). In this case, what would be of interest would be the system's PHB productivity alone and not how much energy would be spent to illuminate it. Considering this, then the operation under a feast regime surges as the best strategy for selecting PMCs for PHB production, due to the culture's higher PHB productivity. Moreover, in a feast regime this high productivity is occurring continuously and simultaneously with cell growth while in the feast and famine regime, besides the lower productivity, the PHB production occurs intermittently alternating with periods of biomass growth. Also, the feast and famine regime may still require the utilization of a second reactor (accumulator) in order to maximize the culture PHA content. Indeed, when a feast and famine regime is applied, it typically requires two reactors for PHB production, the selector SBR for culture selection, and a batch accumulator where the accumulation takes place. The feast regime has advantages in this aspect, because only one reactor is required since the selector SBR is also the accumulating one. This leads to a higher simplification of the process and to a decrease of operational costs.

In addition, the feast regime allowed the accumulation of high PHA contents, up to 60% (Table 5.2), which has the advantage of facilitating the downstream processing during PHA extraction, contributing to a further reduction of the operational costs.

Another positive aspect of the feast regime strategy is that it enabled the selection of a PMC with a high phosphate removal capacity, a feature that was not observed in the PMCs selected under feast and famine regimes. The selector SBR PMC (Figure 5.5A) and more evidently, the PMCs of SBR 1 and SBR 2 (Figure 5.8) were capable of removing in few hours, phosphate amounts similar to those that typically occur in wastewater influents (Metcalf & Eddy, 2003). This is a value added characteristic because it can assure the disposal of a better effluent quality that complies with phosphate discharge limits in case future applications of this system utilize agro-industrial residues/effluents as feedstock.

Despite all these advantages of operating a PMC under a feast regime, the lower PHB production efficiency of the enriched culture is a drawback. If this parameter is analyzed not

from the economical angle, but from the perspective of how efficiently can the PMC cells use energy light to produce PHB, then the PMC operation under the feast and famine regime has clear advantages in this aspect since this culture uses light much more efficiently. It could be the case that if operated under the same light availability of SBR 1, the feast and famine selected PMC could have reached higher PHB productivities.

Summing up all these considerations, the feast regime appears as a promising strategy for selecting photosynthetic PHA accumulating bacteria: 1) it is a simple process that requires only one reactor, 2) PHB is continuously produced with 3) high productivities and it 4) enables a high removal rate of carbon and phosphate. However, it leads to low light utilization efficiency in comparison to feast and famine selected PMCs, and it still needs to be proved its effectiveness under transient illumination conditions.

Therefore future work should focus on long term operation of the SBR under optimized illumination conditions in order to compromise a high PHB production with an increased light utilization efficiency. Moreover, studies under dark/light periods may lead to a further development of this operational strategy for a future utilization of direct sunlight illumination.

5.4 CONCLUSIONS

This work evaluated the possibility of selecting a PHA accumulating photosynthetic mixed culture under a feast regime. Results indicated a possible light limitation in the selector SBR during the stable operation of the culture, leading to low PHB accumulation levels. However, SBR tests showed that the PMC was capable of achieving 60% PHB content when operated with higher light availability. Also, high phosphate removal was observed with this culture, a feature that was not previously observed in other PMCs and that can have a positive repercussion in the SBR effluent quality. Furthermore, microbial analyses indicate a possible alteration in the PMC species composition in comparison to other PMCs selected under feast and famine regimes, with PHA accumulation being observed only in the culture's *Gammaproteobacteria*. In relation to other photosynthetic feast and famine systems, the feast regime enabled the continuous production of PHA with high productivity and requiring only one reactor. These results confirm that the selection of PHA accumulating photosynthetic cultures is not restricted to the application of the feast and famine strategy and that the feast regime strategy is worthy of further investigation for process optimization.

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APPENDIX

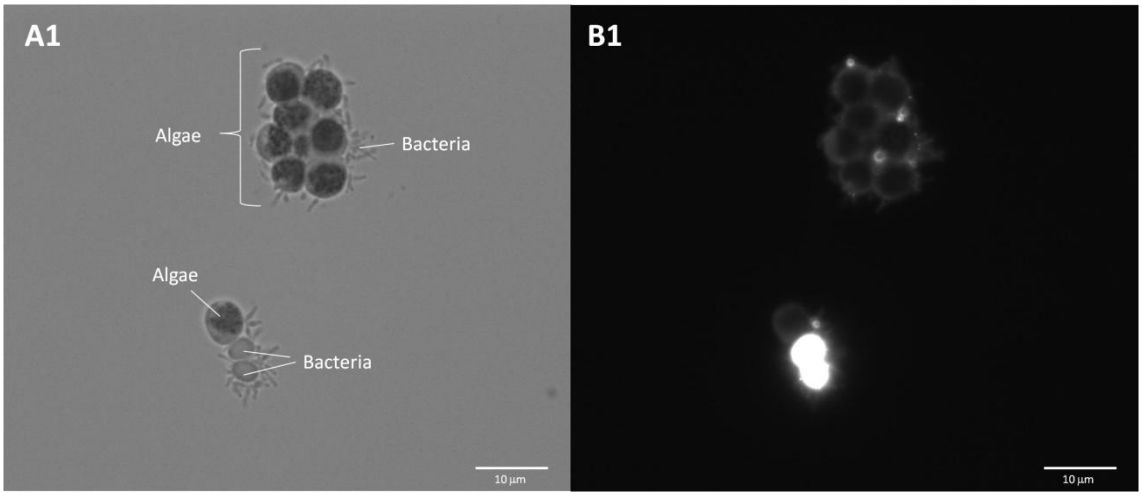

Figure A - Microscopic images of the selector SBR PMC taken during the biomass loss period featuring the algae domain. A1 – Bright field. A2 – Fluorescence images of Nile blue staining.

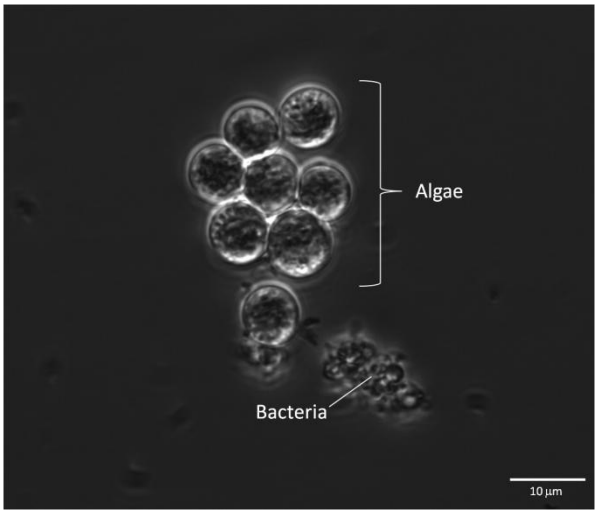

Figure B – Bright field microscopic image of the selector SBR PMC taken during the stably operated period featuring the algae domain.

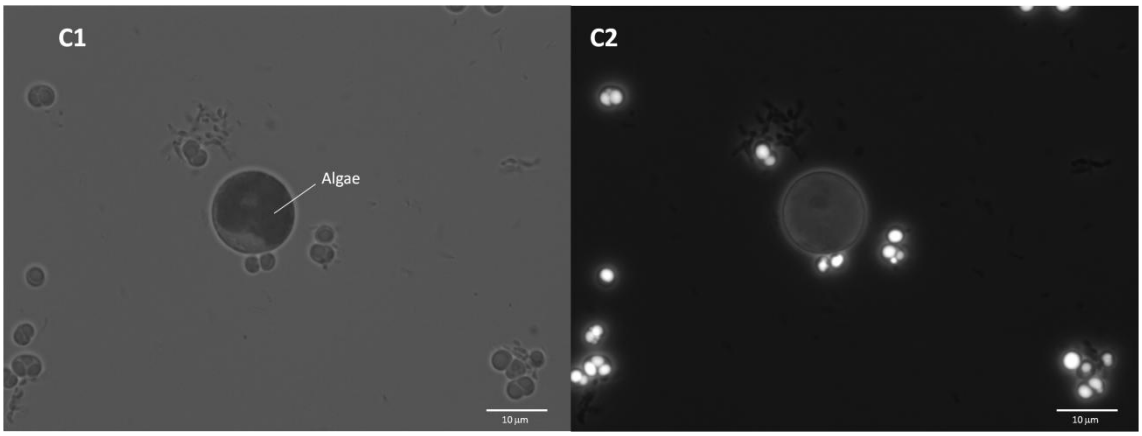

Figure C – Microscopic images of the SBR 1 featuring the algae domain. C1 – Bright field. C2 – Fluorescence images of Nile blue staining indicating PHA granules.

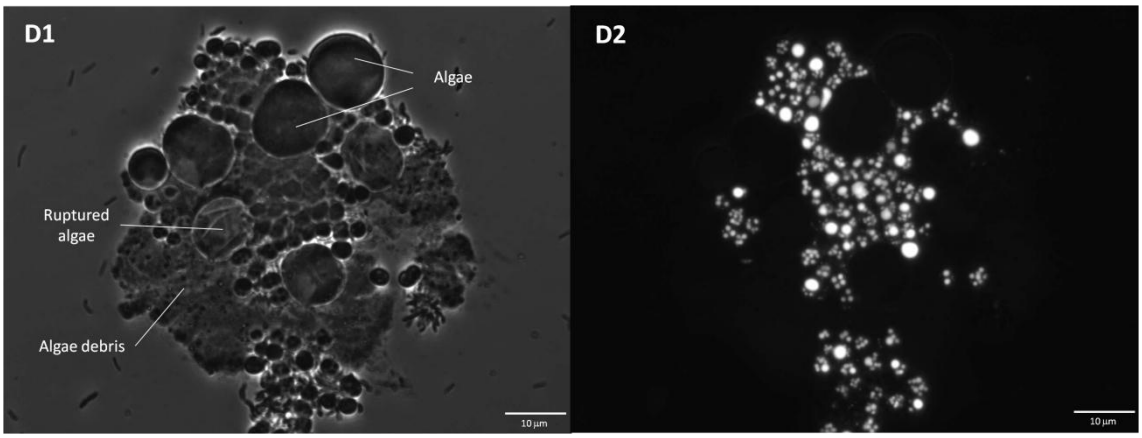

Figure D – Microscopic images of the SBR 2 featuring the algae domain. D1 – Bright field. D2 – Fluorescence images of Nile blue staining indicating PHA granules.

6

Conclusions and future work

The long-established oil based plastic production systems are not environmentally sustainable. They consume finite resources that soon will become depleted and each year, they originate tons of non degradable plastics that accumulate in the natural habitats, polluting the environment. In the near future, new sustainable practices must be quickly exercised in order to produce more ecological polymers. For this reason, a strong effort has been placed in the development of PHA producing systems, given that PHA is a biopolymer that can be produced from renewable feedstocks and it leads to the production of completely biodegradable plastics. In line with this research effort, this work contributed to the development of a new PHA producing process that expanded the possibilities of PHA production by utilizing new organisms and new operational conditions. It explored the photosynthetic bacteria potential for PHA production in open systems, maintaining the advantages of MMCs operation but without the need for aeration. The first results with a photosynthetic mixed culture led to a maximum PHA content of 20% cdw that was achieved with a culture composed of a consortium of bacteria and algae, being the bacterial PHA production mostly dependent on the illuminated environment and not on the oxygen produced by the algae. Furthermore, carbohydrates were used as an additional source of energy for substrate uptake and were restored via PHA degradation, in a cyclic pattern that was very similar to glycogen utilization by GAOs. With these results, this work demonstrated, for the first time, the capacity of photosynthetic mixed cultures to accumulate PHA.

Considering that the major benefit to utilizing a photosynthetic system is the possibility of using direct sunlight, this implies that PMCs must be capable of enduring both the light and dark periods that accompany day and night. By imposing an alternating illumination pattern, results showed that the PMC adaptation to the new illumination conditions led to a decrease in its algae content, maintaining nevertheless its viability and PHA accumulation capacity. Moreover, the algae concentration decrease liberated more light for the remaining organisms, leading to a direct correlation with the increase in culture specific substrate consumption rate. With the diminishment of non-PHA-accumulating organisms, the PMC became further enriched in PHA accumulating organisms and achieved PHA contents of 30% cdw. Though this value presented an increase in relation to the results obtained with continuous illumination, they were obtained in a system that used 8 hour cycles, alternating 4h light/4h dark. For future direct sunlight utilization, this illumination pattern must be changed to 12h light/12h dark in order to simulate a circadian cycle. As a result, the cycle's length needs to be adjusted in future work. Having a 24h cycle with only one feast and famine block per cycle may not necessarily be the best option. Perhaps several feast and famine cycles, of 3 to 4h each, may be necessary during the 12h illuminated period. This point should be addressed in future work focused on optimizing PHA production from PMCs. While the following 12h dark period may not directly contribute to

PHA production, it can possibly affect the culture composition by promoting the selection of other organisms, e.g. GAO-like organisms that accumulate carbohydrates during the light period and consume them in the dark. These organisms could either be PHA accumulators that also store carbohydrates, or could well become competitors to the photosynthetic PHA accumulating bacteria, using their stored carbohydrates for anaerobic growth. Considering that in the present work with 4h light/4h dark a low algae content was observed, it cannot be discarded that a similar occurrence might take place with the circadian illumination. Though a small algae amount promotes higher light availability for PHA accumulating organisms and leads to higher PHA contents, it is important that algae are present in the culture to produce enough oxygen for PHA bacterial consumption during the illuminated famine phases, thus enabling the FF strategy.

In relation to the effect of the substrate feeding composition on the PMC PHA accumulation capacity, results indicated that VFAs were the best substrates for PHA production. Furthermore, the culture was capable of accumulating P(3HB-3HV) co-polymers when fed with propionate, similarly to what occurs with the traditional PHA accumulators MMCs when fed with odd-carbon substrates. This is of significant importance considering that co-polymers tend to present better thermoplastic properties than pure PHB. Also, another interesting finding from these results is that in this acetate enriched PMC, butyrate and propionate consumption accelerated in the presence of acetate. Apparently, acetate derived acetyl-CoA facilitated the internal processing of the taken up propionate and butyrate, thus accelerating their metabolism and consequently, their consumption. This result enforces the importance of co-substrates like acetate being present in the medium in order to guarantee the culture's full substrate uptake potential. Though these tests with different feeding composition were performed with synthetic substrates, they led to an improvement of our understanding of the PMC metabolism. But more importantly, they point toward the future utilization of cheap VFA-rich fermented wastes as feedstock. However, more than just test the utilization of real streams for PHA production, the PMC itself should be subjected in the future to enrichment using fermented wastes as a feed source.

It is expected that such an enrichment using a mixture of VFAs will lead to different bacterial populations, altering the microbial structure of the culture as well as the PHA production profile. It would also be of interest to monitor these population changes and try to link the microbial community structure with the process performance. Thus far, only FISH techniques were used to monitor and identify the PMC's organisms, resulting in a general identification of the organisms as *Alphaproteobacteria* and *Gammaproteobacteria*. In the future, more advanced molecular techniques like pyrosequencing could be used as well to examine the population

changes and identify in detail the culture bacterial and algal composition. This can also be linked with results from Nile blue staining observation of cells' internal PHA granules in order to estimate the contribution of different bacterial groups to the culture's PHA accumulation. Further attention must also be given to the algae domain. Since light/dark periods negatively affect the algae population, it cannot be excluded that the algal fraction could be affected by enriching the PMC with real wastes. The results of this work suggest that algae likely contribute towards oxygen production, thus their presence should not be eliminated, yet they do not contribute towards PHA production, thus their quantity should be maintained at low levels. However, the role of the algal fraction in feast/famine PHA producing PMC systems should be studied in greater detail in order to determine the best operational strategy. Moreover, efforts should be dedicated to the exploration of techniques that allow the quantification of the algae and the bacterial domain in order to assess their prevalence.

Although such interesting results were obtained with feast and famine selected PMCs, it is also true that the utilization of a permanent feast regime emerges as an attractive new line of work. Unlike the feast and famine strategy where organisms are selected for their capacity to grow on accumulated PHA, the feast regime selects organisms for their capability of internally regulating the cell's reducing power through PHA formation. By applying this new strategy, the feast regime enabled the selection of a PMC that could simultaneously grow and accumulate PHA, attaining a maximum PHB content of 60% cdw, the highest value reported for a PMC thus far. Apparently, a new microbial population was selected under this new regime presenting the additional capability of high phosphate removal rates, a feature that was not previously observed in feast and famine selected PMCs and that assures the disposal of a better quality effluent regarding phosphate discharge limits. In spite of such promising results, these are just the first results obtained with a permanent feast strategy that still requires further development. In future work, the culture should be operated with higher light availability while monitoring its population and PHA accumulation. With the long-term operation at high light availability, an accelerated growth of the culture is expected and the increase of the biomass concentration (that would lead to low light availability) may have to be compensated with reductions in the SRT. Also, considering that the feast regime requires the permanent presence of a substrate, it could be hypothesized that the continuous operation of the reactor would be more effective. Future work could evaluate this question as well. However, continuous operation would most likely affect the quality of the effluent, since organic compounds would still be present in the exit stream. This aspect becomes even more relevant if future sunlight illumination is used, which would imply dark periods where the culture would decrease its carbon uptake and potentially decrease the treatment efficiency.

But it is not only the permanent feast strategy that requires further development. The photosynthetic PHA production systems that were operated in this work also need to have their operational parameters optimized. For example, the PMCs of this work were always selected and operated at 30º C and pH 6.5, and as previously referred, temperature and pH, are parameters that besides influencing the organisms selection, they also influence the organisms metabolism. It is then expected that an adjustment of these parameters combined with other optimized parameters may lead to an improvement of the culture PHA accumulation capacity and PHA productivity.

Finally, the ultimate goal of operating a photosynthetic reactor is to use direct sunlight. Therefore, future photosynthetic PHA producing systems will have to operated outdoors and be subjected to the exterior natural conditions. Seasonal and daily variations of the illumination periods and temperature will be the major factors influencing the PMCs stability and process performance. In addition, reactor design will have to be carefully planned in order to properly respond to the practical challenge of using sunlight illumination.

If PMCs can be successfully operated outdoors and using cheap, VFA-rich fermented wastes as feedstock for PHA production, then the photosynthetic PHA production system will address the society's request for a sustainable and alternative plastic production system that comprises the utilization of environmentally sustainable and renewable feedstocks, and the production of biodegradable plastics.